Endocrinological Studies on the Regulation of Reproductive and Metabolic Functions in Trained Yearling Horses

（一歳馬における生殖機能と代謝機能の調節に関する内分泌学的研究）

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Content

Chapter 1

Introduction

1.1 Horse

1.1.1 Horse and interaction with humans

The horse (*Equus caballus*), odd-toed ungulate mammal which has single-digit oval-shaped hoof, is taxonomically classified to the order Perissodactyla and family Equidae (Grubb 2005). Wild horses were likely domesticated by human prior to 4000 BC with the earliest archaeological evidence on approximately 3500–4000 BC (Outram et. al 2009; Matossian 1997). The domestication was completed by 3000 BC and widely distributed throughout Europe continent by 2000 BC (Evans 1992). Domestication of wild horses led to horse breed variations and the different characteristics in horses (Bennett and Hoffmann 1999; Ensminger 1969). Horses are endothermic herbivores which are naturally grazing animals and have 64 chromosomes with 2.7 billion DNA base pairs (Antczak and McConville 2006). Horses can be categorized to hot-, warm- and cold-bloods. These words are widely used for equine terminology to describe in temperament not body temperature of horses. The characteristics of hot-blooded horses such as racehorses show more sensitivity and energy whereas the cold-bloods such as draft horses are quieter and calmer (Belknap 2004). The term of warm blood nowadays means to any cross between cold-blooded and hot-blooded breeds (Belknap 2004) which are used for competition in dressage and show jumping (Price and Shiers 2007). Since the old time until today horses have been used for many purposes by human e.g. leisure activities, sports (racing, show jumping dressage riding, endurance riding and competitions), and working (war, agriculture, horse police,

etc.). Recently, hippotherapy, term are used for different physical, occupational, and speech therapy treatment strategies that utilize equine movement. In hippotherapy, a therapist uses the horse's movement to improve their patient's cognitive, coordination, balance, and fine motor skills, whereas therapeutic horseback riding uses specific riding skills.

1.1.2 Horse age

In equine field, there is the use of terminology to describe horses in various ages as follows:

- Foal is a horse of either sex less than one year old. A nursing foal is sometimes called a *suckling* and a foal that has been weaned is called a *weanling* (Ensminger 1969). Most domesticated foals are weaned at five to seven months of age while weaning in natural wild foals are eight to nine months old. However, the domesticated foals can be weaned at four months with no adverse physical effects (Giffin and Gore 1998).
- Colt means a young male horse under four years old (Ensminger 1990).
- Filly refers to a young female horse under the age of four (Ensminger 1969).
- Yearling refers to horse of either sex that is between one and two years old (Ensminger 1969). In horse racing, these definitions may differ. For example, in the British Isles, Thoroughbred horse racing defines colts and fillies as less than five years old. However, Australian Thoroughbred racing defines colts and fillies as less than four years old (Yuille 2008).
- Gelding means a male horse of any age which is castrated (Ensminger 1969).
- Mare refers to a female horse from four years old and older (Ensminger 1969).
- Stallion is a non-castrated male horse four years old and older (Ensminger 1969).

1.2 Equine growth

1.2.1 Lifespan and life cycle

The lifespan of domestic horse has been reported around 25 to 30 years depending on breed, environment, management and career (Ensminger 1969). Life stage or life cycle of horse can be divided into 4 phases e.g. foal, adolescence, adulthood and geriatrics. There are differences of growth and development among those phases. In foal, 11 months of gestation termed foal was born and most developed rapidly from birth to 12 months of age (Fig. 1.1) attaining 50 to 60% of mature weight and height in the first year and 80% to 90% of mature weight and height by 24 months of age. In yearling horse, in this stage horse's legs almost grow in the length, and the body trunk is filled out. The two year of age, horse will reach his or her adult height and weight. The four years old horse will become adulthood and be completely growth. Aged horse shows signs of aging. This stage horse will have rapid deterioration and become death (Freeman and Topliff 2002).

1.2.2 Endocrinology of growth and metabolism

The development and growth of all living organisms from the simplest single cell to the most complex mammal require intrinsic and extrinsic factors and also need to adapt to environment for maintenance and survival of life. Endocrine hormone is one type of communication systems which cells, tissues and organs of the body used for coordination of their activities. The endocrine hormones are carried by the circulatory system to cells throughout the body where they bind with receptor and initiate many reactions (Guyton and Hall 2006). Some endocrine hormones affect most cells of the body such as growth hormone causing growth in most parts of the body, and thyroxine increasing the rate of many chemical reactions in almost all the body's cell. Other hormones affect only specific tissues

called target tissue, because only these tissues have receptors for the hormone. For example, adrenocorticotropic hormone specifically stimulates the adrenal cortex, causing it to secrete adrenocortical hormones. The multiple hormone systems of the body play a key role in regulating almost all it functions. Besides growth hormone, thyroid hormone and insulin-like growth factor I are the important endocrine hormone which is involved in growth and metabolism (Guyton and Hall 2006).

Fig. 1.1 Development of Thoroughbred horse from foal to yearling in Hidaka Training and Research Center, Hokkaido; (A) 2 days; (B) 2 months; (C) 4 months; (D) 12 months of age.

1.2.2.1 Thyroid hormone

The thyroid gland secrets two significant hormones, thyroxine and triiodothyronine, commonly called T4 and T3, respectively in response to the stimulations of thyrotropin-releasing hormone (TRH) from hypothalamus and thyroid-stimulating hormone (TSH) from anterior pituitary gland. Both of these hormones have the profound effect of increasing the metabolic rate of the body and promote growth and development (Guyton and Hall 2006; Chen and Riley 1981; McGuire et al. 1991). All of the circulating T4 is derived directly from thyroid gland. Only 10%-20% of the circulating T3 is secreted directly from thyroid gland (Reed et al. 2004). Nevertheless, most of T4 is finally converted to T3 in the tissues. Over 99% of thyroid hormones circulating in blood bind to several type of plasma proteins which synthesized by the liver (Guyton and Hall 2006). Thyroid hormone combines mainly (70%) with thyroid hormone-binding globulin (TBG), thyroxin-binding prealbumin (TBPA, transthyretin) and albumin, bind to the lesser degree. In horse, the percentages of circulating T4 bound to TBG, TBPA and albumin were found to be 61%, 22% and 17%, respectively (Larsson et al. 1985). T3 is bound to TBG and albumin but not to TBPA (Reed et al. 2004).

The thyroid hormone receptors belong to the superfamily of nuclear receptors that work as transcription factors. The two types of thyroid hormone receptors are TR-α and TR-β. T3, whether directly transported into the cell or derived intercellularly from T4, is considered to be the effector hormone in target cells. The thyroid receptors interact with specific DNA sequences (T3-response elements), regulating gene expression. Growth and thermogenesis depend on the gene expression of thyroid hormones. Thyroid hormone decreases the expression of the α- and β-subunit genes of TSH and the TRH gene. From its effects on gene expression, T3 actions result in thermogenesis; increased oxygen consumption; increased

protein synthesis; increased metabolic rate; increased carbohydrate absorption and glucose metabolism; increased lipid metabolism, increased sensitivity of adipose tissue to lipolysis, increased neural transmission; and cerebral and neuronal development in young animals (Kaneko 1989).

1.2.2.2 Insulin-like growth factor I (IGF-1)

The insulin-like growth factor I is the somatomedin C, (small protein from the liver) which has effect on growth similarly to the effects of insulin on growth. Therefore, somatomedins are also called insulin-like growth factors. IGF-1 mainly secreted by liver is induced by growth hormone from pituitary gland. The IGF-1 concentration in the plasma normally follows closely the rate of secretion of growth hormone. Some aspects of the somatomedin hypothesis are still questionable. One possibility is that growth hormone can also cause formation of enough IGF-1 in the local tissue resulting in the local growth. It is also possible that growth hormone itself is directly responsible for increased growth in some tissues and that the somatomedin mechanism is an alternative means of increasing growth but not always a necessary one (Guyton and Hall 2006).

Several researches have been conducted about IGF-1 functions in various animal species. For example, IGF-1 plays role to promote body growth in animals including the period of embryonic development (Doherty et al. 1994; Herrler et al. 1998; Fabian et al. 2004; Wang et al. 2009). It is well-known as endocrine and autocrine/paracrine factor in local tissue such as testis and epididymis of various species (Brokaw et al. 2007; Yoon and Roser 2010) and is involved in steroidogenesis in rat, mice and pig Leydig cells (Lin et al. 1986; Kasson and Hsueh 1987; Wang and Hardy 2004). In horses, IGF-1 and its receptor has immunolocalized stronger in post-pubertal than pre-pubertal stallions (Yoon and Roser 2010) and also improve sperm longevity of equine spermatozoa (Champion et al. 2002).

Furthermore, IGF-1 and its receptor are localized in equine Leydig cells in aged dependency of stallions (Yoon et al. 2011) and also promote follicular growth and enhanced ovarian activity in mares (Derar et al. 2005; Hammond et al. 1991).

1.2.3 Other hormone (Cortisol)

Cortisol is steroid hormone which has glucocorticoid and mineralocorticoid activities. The principal glucocorticoid is secreted by adrenal cortex. Cortisol is very potent and accounts for 95% of all glucocorticoid activity. The key for control of cortisol secretion is the excitation of the hypothalamus by different types of stress which stimulate anterior pituitary gland resulting in adrenocorticotropic hormone (ACTH) releasing. The effects of cortisol are quoted in several aspects as follow; reduced in cellular protein stores in body cells except those of liver, increased the liver and plasma protein, increased blood amino acids, promoted mobilization for fatty acid from adipose tissue, reduced the inflammation in the body and blocked the inflammatory response to allergic reactions (Guyton and Hall 2006).

In addition, cortisol has been used as the indicator of stress. Almost any type of physical or mental stress can lead within minutes to greatly enhanced secretion of ACTH and consequently cortisol as well, often increasing cortisol secretion as much as 20-fold. Pain stimuli, caused by any type of physical stress or tissue damage are transmitted first upward through the brain stem and eventually to the median eminence of the hypothalamus. Corticotropin-releasing hormone (CRH) is secreted into hypophysial portal system. Within minutes the entire control sequence leads to large quantities of cortisol in the blood. For mental stress, it can cause an equally rapid increase in ACTH secretion. This is believed to result from increased activity in the limbic system, especially in the region of the amygdala and hippocampus, both of these then transmitting signals to the posterior medial hypothalamus (Guyton and Hall 2006).

1.3 Equine reproduction

1.3.1 Estrous cycle in mare

Both mare and stallion is seasonal polyestrous breeder. In breeding season, spring and summer, the ovary and testes are active. During this period, non-pregnant mare can show a series of repeated spontaneous estrous cycles and recurring ovulation. Each estrous cycle is 21 to 22 days (range, approximately 18 to 24 days), with estrus comprising 4 to 7 of these days and diestrus remains relatively constant at 14 to 15 days (Davies Morel 2015).

1.3.2 Endocrine control of reproduction

The endocrinological control of the reproduction on both female and male horses is regulated by the pineal gland and hypothalamic-pituitary-gonadal (HPG) axis. Day light is perceived by photoreceptor on horse retina, and then the neurosignals are sent to the pineal gland in the base of the brain. Normally, melatonin is produced nocturnally by the pineal gland, dominates the reproductive system by inhibiting HPG axis's activity. When day length increasing, inhibition of the axis is removed allowing GnRH produced by the hypothalamus to reach anterior pituitary gland through portal blood vessels. The pituitary hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which are produced by gonadotrophs, are released and then act to the gonads in male and female horses. The FSH stimulates the follicular growth resulting in estrogen secretion and heat in female whereas promote spermatogenesis in male testes. LH released in a pulsatile manner, stimulates further development of growing follicles and also leads to ovulation in female horses while stimulates the testosterone secretion from Leydig cells of testes in male horses. After ovulation, LH helps to promote corpus luteum (CL) formation which stimulates progesterone, relaxin and inhibin productions (Tortora and Derrickson 2008; Davies Morel 2015). In

controlling of this pathway, the releasing and inhibiting hormone of hypothalamus will stimulate and suppress the release of pituitary hormones. Also, the hormone secretion of pituitary gland will be regulated by negative feedback from gonadal hormones (see Fig. 1.2).

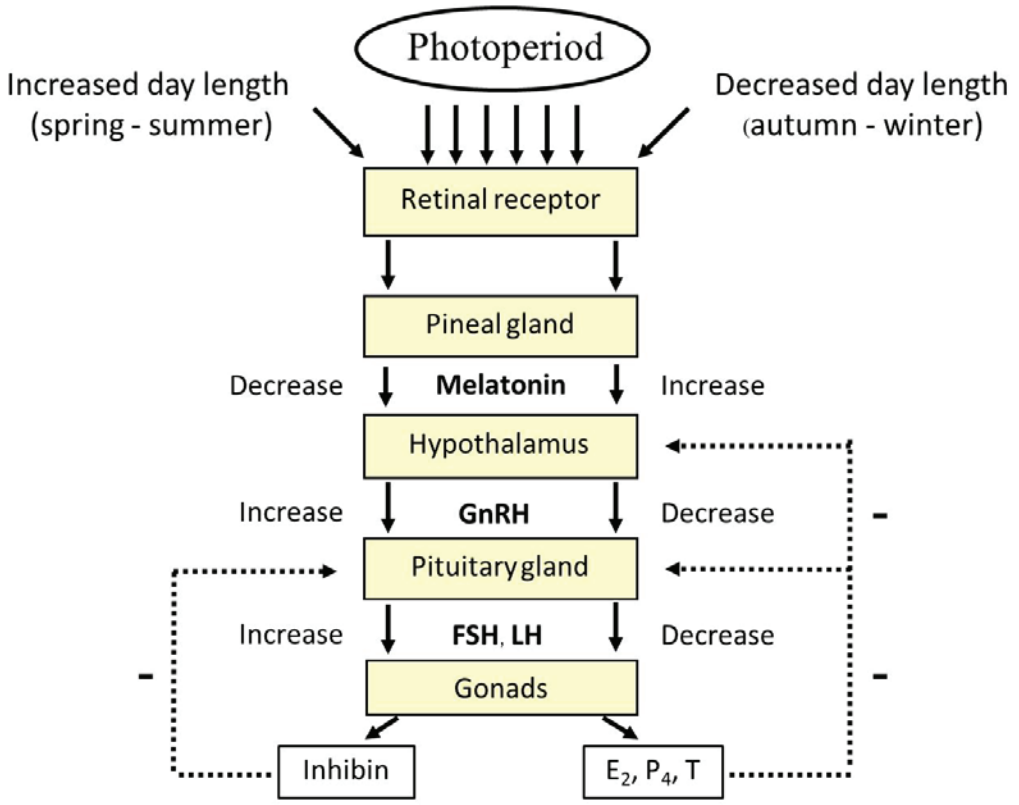


Fig. 1.2 The hypothalamic-pituitary-gonadal (HPG) axis. E_2: estradiol, P_4: progesterone and T: testosterone**.**

1.3.3 Onset of puberty

Onset of puberty is influenced by many factors; endocrine hormone, nutrition, environment, etc. Some theory said that the onset of puberty is regulated by the maturity of the hypothalamic-pituitary-gonadal axis interconnections rather than by the pituitary not producing gonadotropins or by gonadal insensitivity (Squires 1993). The pre-pubertal animals maintain LH pulses in low amplitudes and frequencies and their GnRH pulse generator are very sensitive to negative feedback effects of estradiol and testosterone. At the onset of puberty, the increases in frequency of LH pulse leads to follicular development to more advanced stage resulting in secretion of higher estrogen concentration and stimulation of uterine growth. LH pulse increases and reaches a threshold for enhancing the ovarian growth to pre-ovulatory stage, estrogen induces a pre-ovulatory stage of gonadotropins and ovulation occurs (Squires 1993). Moreover, nutrition condition, body composition and metabolic factors may have a stimulatory or inhibitory effect on puberty in animals and human of sufficient age and weight (Kirkwood and Aherne 1985; Hopwood et al 1990). Photoperiod has influenced on the onset of puberty in seasonal breeder such as horse. Naturally, horse gives birth in the spring season with plenty of food sources. The puberty of horse will occur in the second spring of life at 12 to 15 months of age approximately (Wesson and Ginther 1981). In horse, fillies will reach puberty at 10-25 months of age (Wesson and Ginther 1981; Camillo et al. 2002). Spring-born fillies show heat as yearlings, and has first ovulation during late spring or early summer while those born later in the year generally do not cycle until they are two years old (McGing et al. 2016). The puberty in colt has been reported between 14-24 months of age by determination of semen characteristics (Skinner and Bowen 1968). However, the age of puberty has variation among breed of horse.

1.3.4 Photoperiod and reproduction

A photoperiod is the duration of light within a 24-hour period which plant or animal is exposed. Generally, most living things have their biological clock which use for response to the light and dark cycle of 24-hour rhythms in order to coordinate their biology and behavior with daily environmental changes. Photoperiod which is an important environmental factor regulate circadian cycles (Goldman and Nelson 1993). Besides the suprachiasmatic nucleus (SCN) in hypothalamus, retina and pineal gland is the internal clock of vertebrates (Kacsoh 2000).

1.3.4.1 Pineal gland-melatonin and regulation on reproduction

Pineal gland which has been viewed as a third eye (Eakin 1973), lies in the epithalamus, near the center of the brain, between the two hemispheres, tucked in a groove. The pineal gland produces melatonin which modulates sleep patterns in both circadian and seasonal cycles (Macchi and Bruce 2004). The darkness stimulates melatonin production while light will inhibit (Axelrod 1970; Lowrey and Takahashi 2000). Light perceived by photoreceptor in the retina of eye and pass as electrical signals to the SCN through the nerve fibers. Then, the nerve fibers relay the electrical impulses (daylight information) from the SCN via the paraventricular nuclei (PVN) in the hypothalamus and the spinal cord to the superior cervical ganglia (SCG) of sympathetic nervous system, and finally reach to the pineal gland (Yu and Reiter 1993). In pineal gland, melatonin is synthesized from tryptophan, amino acid, which converted to serotonin and then catalyzed by two specific enzymes. After converting by N-acetyltransferase, N-acetyl serotonin eventually converted to melatonin (Reiter 1980). In mammals, circulating melatonin concentrations increase in the dark time whereas become lowest during daylight (Kacsoh 2000; Brennanet al. 2007).

1.3.4.2 Light supplemental manipulation in horse

Horse is long-day animal with the highest reproductive functions in long-day season but shows anestrous or transient subfertility during short-day period. Artificial light is commonly performed in horses for advance onset of breeding season or enhance the anovulation during winter anestrus and vernal transition in non-breeding season. Accordingly, the differences of latitude lead to large variation of day length. At equator, daylight is 12 hours per day constantly. Temperate climate zone or the poles have fluctuation of photoperiod between summer and winter season. The minimal length of light exposure necessary has not been critically established, but field experience indicates that 14 to 16 hours of light stimulation (artificial plus natural light) per day is adequate (Brinsko et al. 2011). The lighting programs have traditionally been required a minimum of 8 to 10 weeks for response, mares in the northern hemisphere are exposed to the lighting system by December 1 to establish normal cyclic activity by mid-February. For individual stall-lighting systems, mare should be within 7 to 8 feet of a 200-watt light bulb to provide adequate light exposure and the stall should have sufficient window space to permit the same exposure during daylight (Kenney et al. 1975). The intensity of light, 107 lux approximately using the 100 watt bulb in the center of 12 x 12 feet horse stall is sufficient to improve reproductive function (Sharp and Cleaver 1993). However, various lighting programs suggested best results are obtained when the supplemental light is either added to the end of the day or split and added to both the beginning and the end of the day, instead of adding the supplemental light only at the beginning of the day (Sharp and Cleaver 1993). Recently, the manipulation of light supplementation using 100 watt white bulb in the horse stall (3.6 x 3.6 meter) providing of 14.5 hour light and 9.5 hour dark help to advance molting of winter to

summer coats, induce early ovulation and promote growth in yearling horses (Nambo et al. 2010; Kunii et al. 2015; Suzuki et al. 2015; Harada et al. 2015).

1.3.5 Endocrinology of reproduction in horse

1.3.5.1 Prolactin

Prolactin, one important pituitary hormone plays major role in lactation, hair shedding and reproduction. Prolactin is secreted by pituitary gland in higher level during long daylength period but lower in short-day period on both mares and stallions. In mares, prolactin and its receptor are found in follicular fluid, follicles and CL (Daoud and Ezzo 2014; Henderson et al. 2006; Yoon and Roser 2010). In male horses, prolactin involved in testosterone secretion via the stimulation of LH receptor expression in rodent Leydig cells (Hair et al 2002). Moreover, it is thought to be responsible for changes in metabolic rate, increasing the efficiency of food conversion during the winter months with evidence in the native breeds of horses (Argo and Smith, 1983; Morley et al. 1983; Evans et al. 1991).

1.3.5.2 Estradiol and progesterone

Estradiol-17β which is sex steroid hormone and the most potent in three estrogens (Guyton and Hall 2006), produced from cholesterol by interrelationship between the theca and the granulosa cells within developing follicle of ovary. The theca cells convert cholesterol to testosterone, which diffuses across to the neighbouring granulosa cells, where it is converted to estradiol-17. This final conversion within the granulosa cells depends upon the enzyme aromatase, whose activity is FSH-dependent. Estradiol-17β has various effects on several parts of body. Regarding the reproduction in mare, estradiol-17β is secreted into the blood circulation and 24-48 hours prior to ovulation reaches a peak of 10–15 pg/ml. Then, the levels will drop to basal levels immediately after estrus. The increase of estradiol-

17β concentration is responsible for the behavioral changes in the mare showing estrous sign or heat and accepting of copulation by stallion (Davies Morel 2015). In male horse, estradiol-17β is derived from testosterone by Sertoli cell of testes probably essential for spermatogenesis (Guyton and Hall 2006).

Progesterone is another sex steroid hormone which is synthesized by the ovary. After ovulation, CL forms within the collapsed follicle lumen. The luteal tissue is derived from the old theca cells. Progesterone is secreted by CL so that the level of progesterone rises after ovulation within 24–48 hours. Maximal progesterone concentrations reach to 10 ng/ml after 5-6 days post-ovulation and are maintained until day 15-16 of estrous cycle. If the mare has not conceived, progesterone levels will drop dramatically 4-5 days prior to the next ovulation to give basal levels again during estrus (Davies Morel 2015). Progesterone promotes uterine endometrium in preparing of uterus for implantation and also helps to increase secretion in fallopian tubes to nourish the fertilized ovum before implantation (Guyton and Hall 2006).

1.3.5.3 Testosterone

Testosterone, sex steroid hormone is secreted by Leydig cell of the testes. The Leydig cells are located in the intertubular spaces or interstitial tissue of the testis and are responsible for testosterone production by controlling of LH (Amann 1981). LH is produced in a pulsatile fashion. Therefore such a pulsatile release of testosterone means that a single blood sample for the hormone can give erroneous results; a hormone profile taken over a period of time and averaged is a much more accurate indication of true testosterone levels (Amann 1993). The FSH has been known as hormone to start the process of spermatogenesis, developing spermatogonia to secondary spermatocytes. Then, testosterone completes from secondary spermatocytes to spermatozoa which are ready for passage to the epididymis for

maturation (Davies Morel 1999). Furthermore, testosterone controls the development of male sex organs in the fetus or neonate, pubertal changes and maintains function of the accessory sex glands. Moreover, it is also responsible for male libido and sexual behavior in stallion. In addition, testosterone involves in growth acceleration and also muscular development (Irvine et al., 1986; Flink, 1988).

1.4 Objectives of the study

Regarding growth and reproduction, some connection among thyroid hormone, prolactin and reproductive organs have been reported (Evans et al 1991; Kunii et al 2015; Suzuki et al 2015). However the definite mechanism is still being unclear in young horses. Therefore, the study in this thesis covers a range of studies in development, growth and reproduction by investigation in yearling horses.

The objectives of the study were as follows:

a) To establish a novel radioimmunoassay system that can provide the reasonable total thyroxine values in Thoroughbred trained yearling horses.
b) To determine changes in body physical growth, metabolic and reproductive hormones in Thoroughbred trained yearling horses and compare those changes between Hokkaido and Miyazaki horses under natural condition.
c) To investigate the effect of light supplementation on changes in body physical growth, metabolic and reproductive hormones in Thoroughbred-trained yearling horses which were raised in different climate; the north Hokkaido and the south Miyazaki of Japan.
d) To compare body physical growth, metabolic and reproductive hormones between Hokkaido and Miyazaki horses under light supplementation condition.

Chapter 2

General methodology

2.1 Animals

All procedures in the studies were carried out in according with approval of the Institutional Animal Welfare and Experiment Management Committee of Japan Racing Association (JRA) Hidaka Training and Research Center.

Thoroughbred trained-yearling horses from 1 year of age to less than 2 years of age raised in two facilities of JRA (Fig. 2.1) which were located in the north; Hidaka Training and Research Center in Hokkaido (latitude 42.2° and longitude 142.8°) and the south; Miyazaki Training Yearling Farm in Miyazaki prefecture (latitude 31.9° and longitude 131.4°), were used to be subjects of the studies for two year seasons from 2012-2013 and 2013-2014. Total 160 yearling horses were randomly divided into two groups as follow:

- Control group

  The subjects in control group composed of 25 Hokkaido yearlings; 12 colts and 13 fillies, and 22 Miyazaki yearlings; 10 colts and 12 fillies. All horses in control groups only exposed to natural light without artificial light supplementation throughout experimental periods.

- Light supplementation (LS) group

  The subjects in LS group consisted of 91 Hokkaido yearlings; 44 colts and 47 fillies, and 22 Miyazaki yearlings; 11 colts and 11 fillies. All horses in LS groups received supplemental lighting throughout experimental periods.

Hidaka Training and Research Center, Hokkaido

Miyazaki Training Yearling Farm, Miyazaki

Fig. 2.1 The facilities of JRA in Hokkaido and Miyazaki prefectures, Japan.

2.2 Light supplementation program

In light treatment groups, artificial light supplementation was conducted by using timer-linked 100 watt white light bulb which was set in the ceiling of the horse stall (3.6 x 3.6 m) (Fig. 2.2). The light supplementation was performed 2 times per day as follow;

- At morning, starting before sunrise from 5.30 h to 8.00 h
- At evening, starting before sunset from 16.30 h to 20.00 h

The photoperiod was extended equally as summer in 14.5 h daylight and 9.5 h of dark period (Northern Hemisphere) from December 25th to April 16th in each year for two year seasons.

Fig. 2.2 Individual stall-light supplementation.

2.3 Diets and exercise program

All horses were fed by hay (*Poa pratensis*), oats, and pellet feed (JRA original 10, NOSAN Corporation, Kanagawa, Japan) containing vitamin and trace mineral supplementation in each individual stall 4 times per day. Water was provided *ad libitum*. Through the end of December, Miyazaki horses were let to pasturing and fresh grass grazing daily while Hokkaido horses were pastured in small paddocks and ate dried Timothy grass. For winter pasturing since January, hay and water by heated automatic waterer were available for free access individually by Hokkaido and Miyazaki horses.

Training system was conducted in accordance with JRA traditional training programs for growing horse. Both Hokkaido and Miyazaki horses, horses warmed up in a walking machine for 30 min/day through the end of January while by trotting 800 m in February to March. Low- and high-intensity training were performed by canter and gallop on 800 m, 1600 m flat-track and 1000 m slope courses in Hokkaido (Fig. 2.3A), and 1600 m flat-track course in Miyazaki (Fig. 2.3B), then cooled down by walking.

Fig. 2.3 Slope course in Hidaka Training and Research Center (A) and flat-track course in Miyazaki Training Yearling Farm (B).

2.4 Method of hormone extraction

2.4.1 Extraction for insulin-like growth factor I (IGF-1)

Prior to measurement of IGF-1, the extraction of sample and standard had been required. “Acid ethanol cryo-precipitation”, a common procedure for extraction, was modified from the original method as previously described (Daughaday et al. 1980). Standards or samples (100 μl) with the acid ethanol mixture (400 μl), which was prepared with 2 M HCl and 99.5% ethanol in a ratio of 12.5%:87.5% (v/v) were mixed in 12 x 75 mm glass tubes and incubated at room temperature for 30 min. After centrifugation at 4°C, 2100 x g for 30 min, the supernatant was decanted, neutralized with 0.855 M Tris (hydroxymethyl) aminomethane (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) solution at a ratio of 5:2, mixed thoroughly, and then stored at -20°C for 1 hr. After storage, all tubes were immediately centrifuged at 4°C, 2100 x g for 30 min. The supernatants were collected into fresh tubes and diluted with gelatin-PBS (phosphate buffered saline) which was composed of 0.05 M PBS containing 0.1 % sodium azide (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.1% gelatin and 0.1% Triton X-100 (polyoxyethylene octylphenylether), pH 7.4 (Sigma-Aldrich Co. LLC.) to achieve at final solution, and then stored at -20°C until assayed. Herein, the final extracted solution (the supernatant diluted with gelatin-PBS) needed to be calculated for appropriate dose in order to obtain hormone levels which were expected in different age or status of each species, and adjusted to 50 μl/tube for RIA, which was typically in the middle of the standard curve. The protocol of extraction for IGF-1 is shown in Fig. 2.4.

2.4.2 Extraction for cortisol

Sample (50 μl) mixed with 1% bovine serum albumin; BSA (Sigma-Aldrich Co. LLC) 350 ul in total as 400 ul or serial standard solutions in 1% BSA (400 μl) were prepared into glass

tube (13 x 100 mm), and added 2 ml diethyl ether (Dojindo laboratories, Kumamoto, Japan). After mixing for 3 min, all tubes were bathed in dry ice (99.5%) ethanol until water phase freezing (white spike appearance). The ether phase was decanted into fresh glass tube (12 x 75 mm) and was dried at 60°C in chamber. After cooling, wall of the tubes were rinsed with 0.5 ml ether and were then dried at 60°C in chamber. All tubes were reconstituted with 1% BSA. The protocol of extraction for cortisol is shown in Fig. 2.5.

2.5 Radioimmunoassay (RIA) of hormones

2.5.1 RIA of prolactin

Serum or plasma concentrations of prolactin were measured by the equine prolactin RIA as previously described (Mizukami et al. 2015), using rat antisera against equine prolactin (Lot No. AFP-261987), purified equine prolactin (Lot No. AFP-8794B) for radioiodination, and equine prolactin (Lot No. AFP-7730B) for reference standard. The protocol for RIA is shown in Fig. 2.6.

2.5.2 RIA of immunoreactive (ir)-inhibin

Serum or plasma concentrations of ir-inhibin were measured by the bovine inhibin RIA as previously described (Hamada et al. 1989), using a rabbit antiserum against bovine inhibin (Lot No. H-1), purified 32-kDa bovine inhibin for radioiodination and standard. The protocol for RIA is shown in Fig. 2.6.

2.5.3 RIA of IGF-1

Serum or plasma concentrations of insulin-like growth factor I were measured by the RIA as previously described (Mizukami et al. 2015), using rabbit anti-serum against human

IGF-1 (Lot No. AFP4892898) and recombinant human IGF-1 (Lot No. 090701) for radioiodination and reference standard**.** The protocol for RIA is shown in Fig. 2.6.

2.5.4 RIA of cortisol

Serum or plasma concentrations of cortisol were measured by RIA system as previously described (Arai et al. 1995), using rabbit anti-cortisol (Lot No. HAC-AA71-02RBP85), Cortisol-3-CMO-BSA (Cat. No. 80-IC20) for radioiodination, and Hydrocortisone (Cat. No. H-4001) for reference standard. The protocol for cortisol RIA is shown in Fig. 2.7.

2.6 Fluoroimmunoassay (FIA) of hormones

2.6.1 FIA of estradiol-17β, progesterone and testosterone

Serum or plasma concentrations of estradiol-17β, progesterone and testosterone were measured by time-resolved fluorescence immunoassay using commercial kit (DELFIA). Rabbit antiserum against estradiol, progesterone and testosterone; estradiol-, progesterone- and testosterone-europium tracer; and anti-rabbit IgG (goat) were used for assaying. Fluorescence was measured using the time-resolved fluormeter (1420 multilabel counter). The protocol for estradiol-17β, progesterone and testosterone FIA are shown in Fig. 2.8-2.9**.**

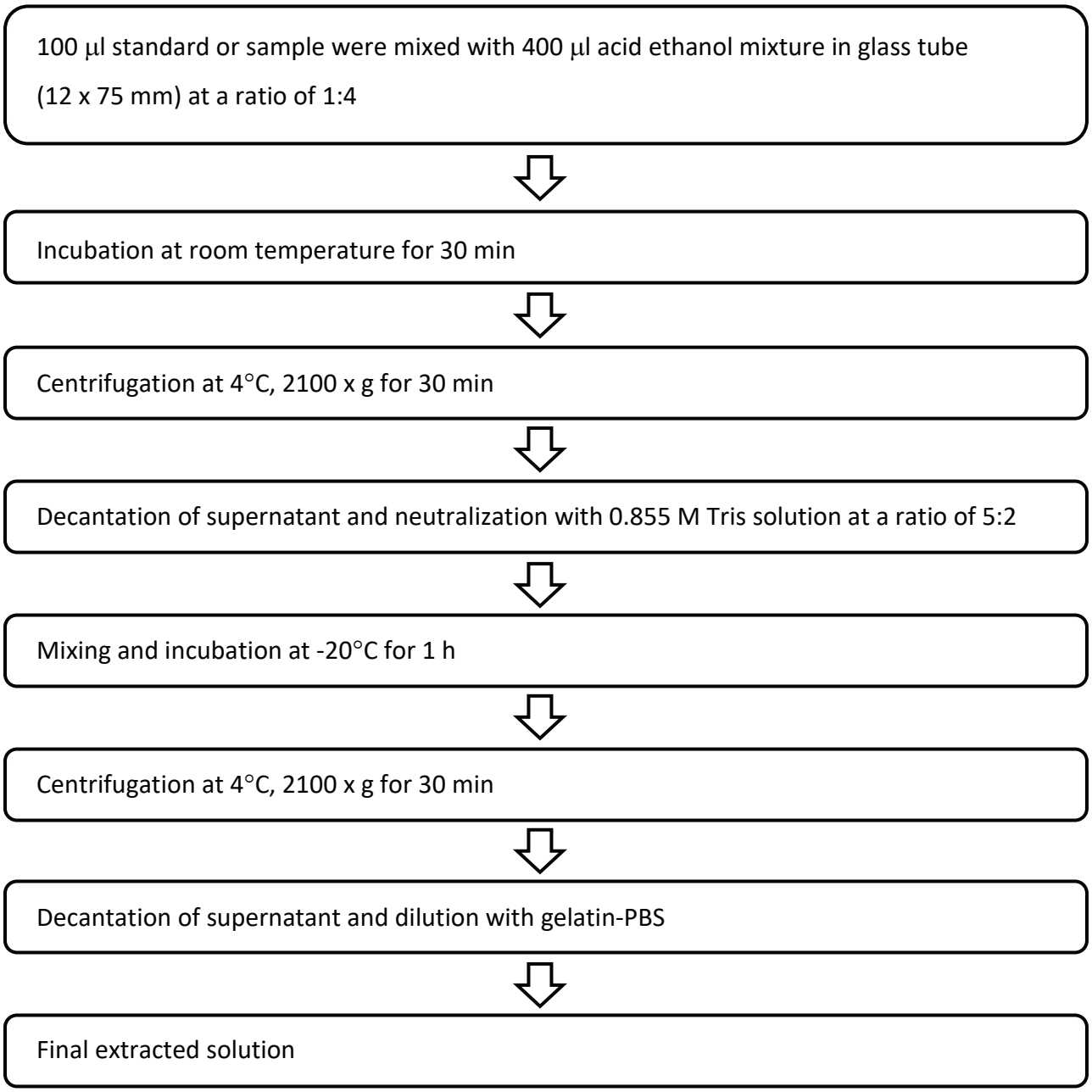


Fig. 2.4 Extraction procedure for insulin-like growth factor I

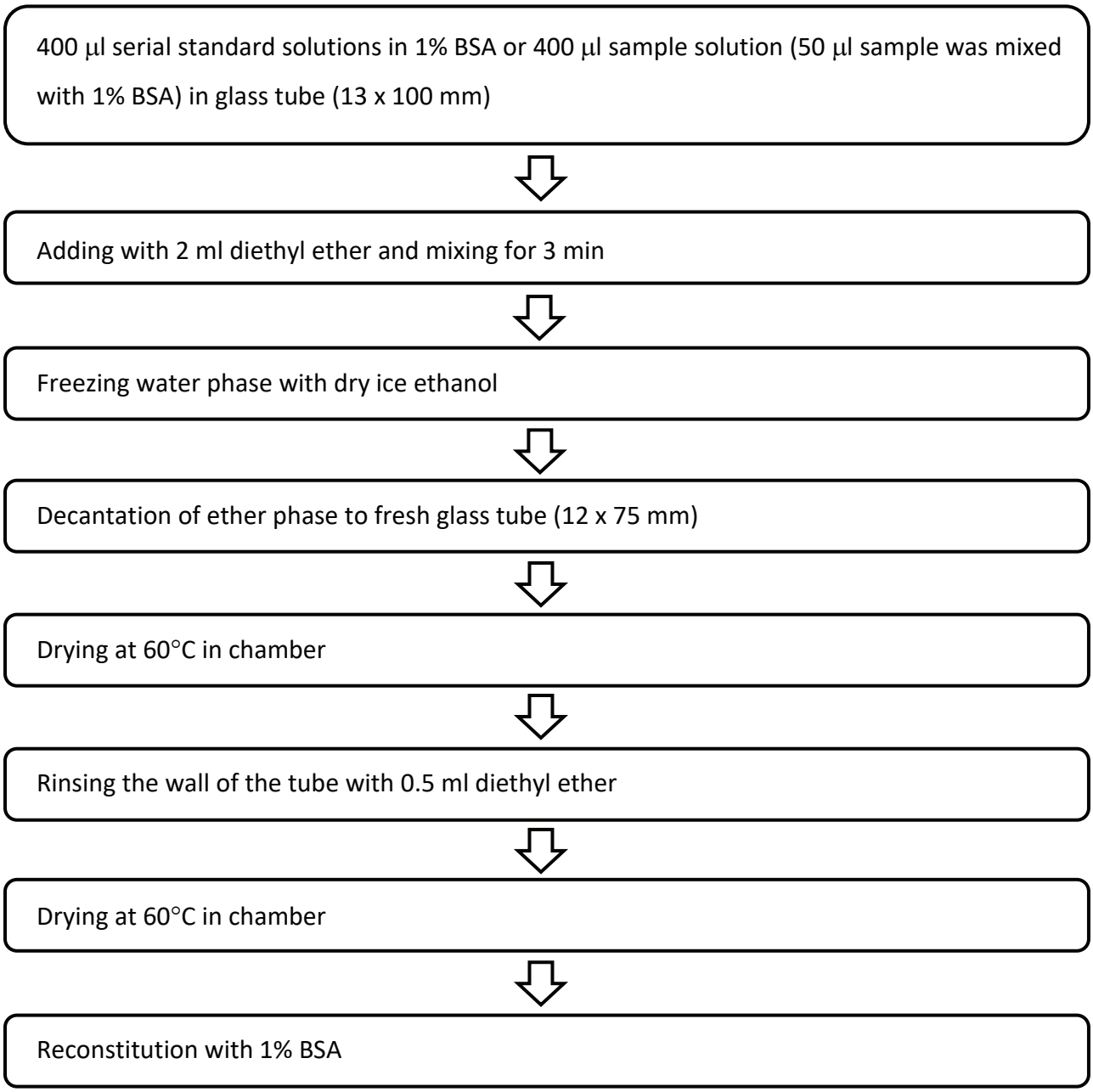
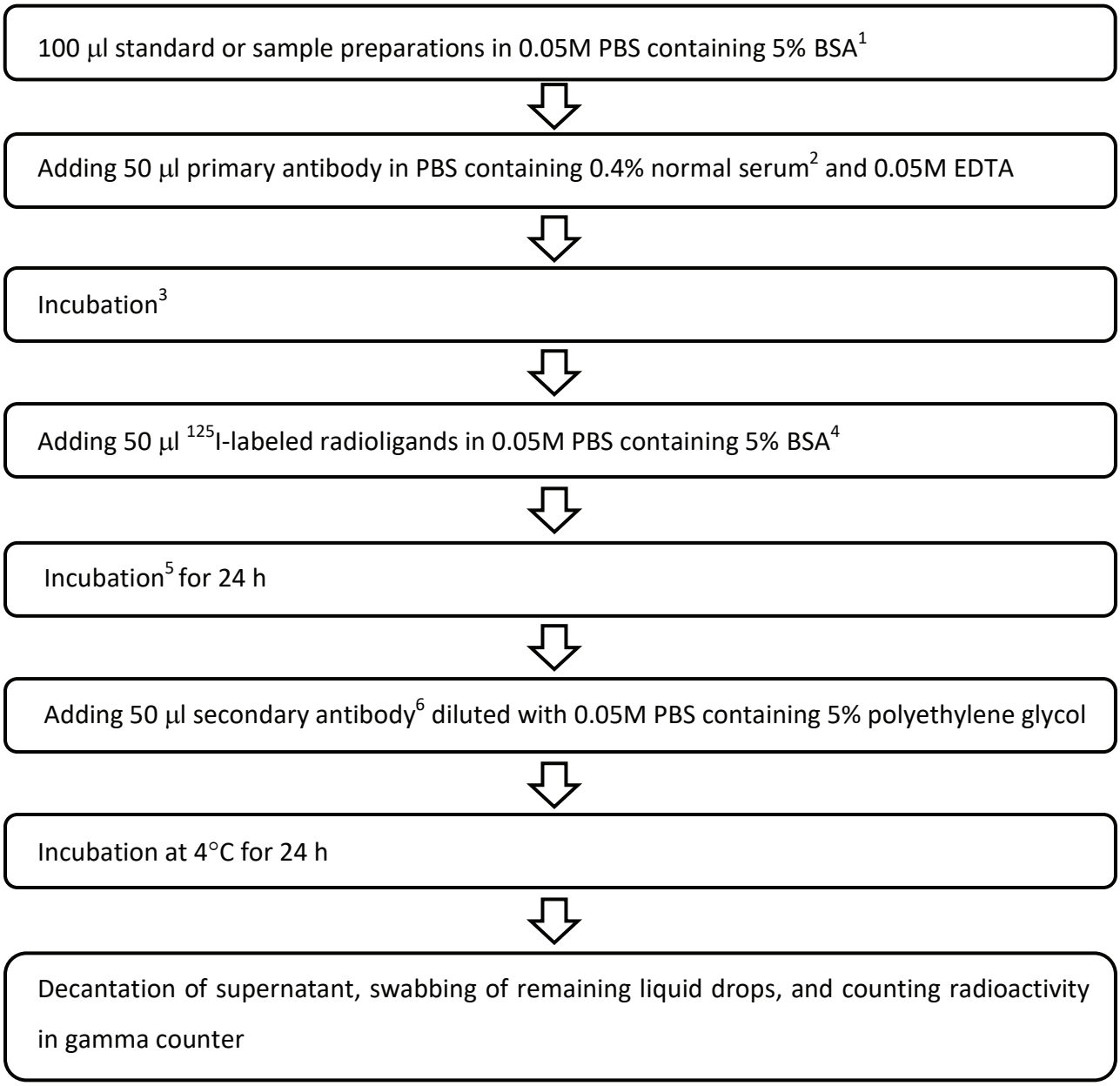


Fig. 2.5 Extraction procedure for cortisol



[1] SPS (mixture of acidic ethanol, Tris, and gelatin-PBS at ratio of 2:1:2) for IGF-1

[2] Normal rat serum for PRL, and Normal rabbit serum for ir-inhibin and IGF-1

[3] 4°C, 24 h for PRL; 37°C, 24 h for ir-inhibin; and room temperature, 1 h for IGF-1

[4] Gelatin-PBS for IGF-1

[5] 4°C for PRL and IGF-1, and 37°C for ir-inhibin

[6] anti-rat gamma globulin for PRL, and anti-rabbit gamma globulin for ir-inhibin and IGF-1

Fig. 2.6 Procedure of radioimmunoassay for peptide or protein hormones

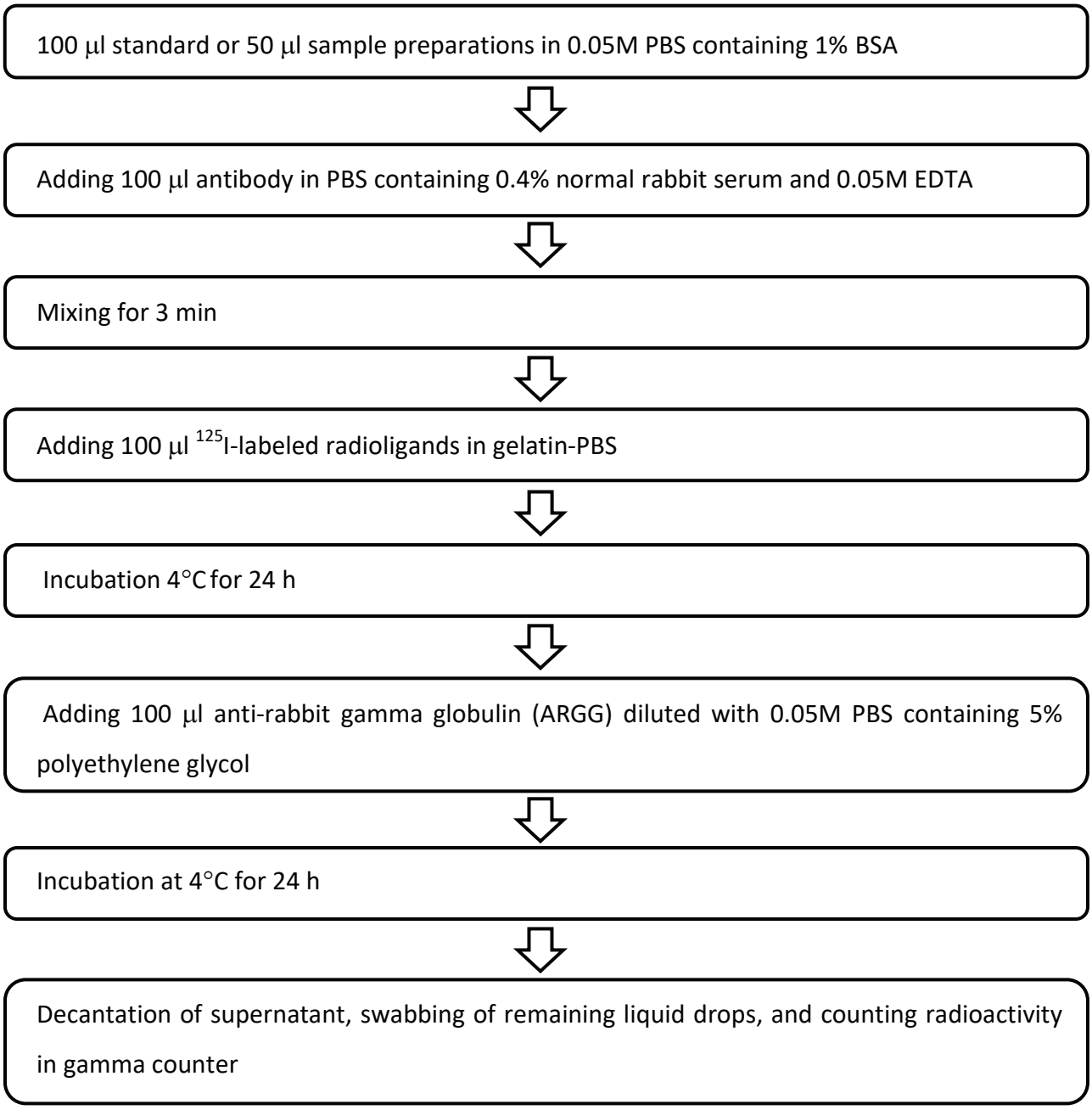


Fig. 2.7 Procedure of radioimmunoassay for cortisol

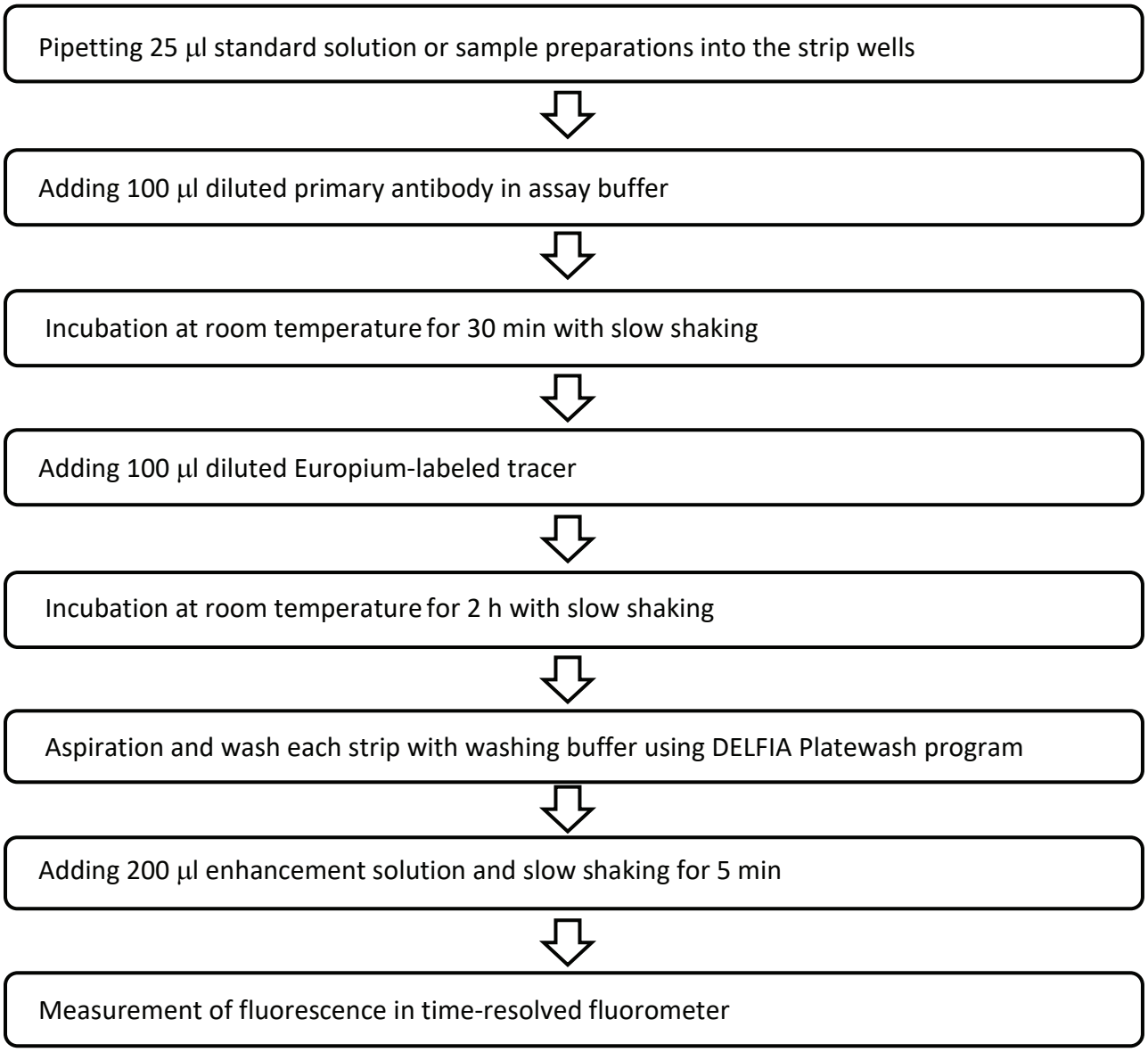


Fig. 2.8 Procedure of fluoroimmunoassay for estradiol-17β

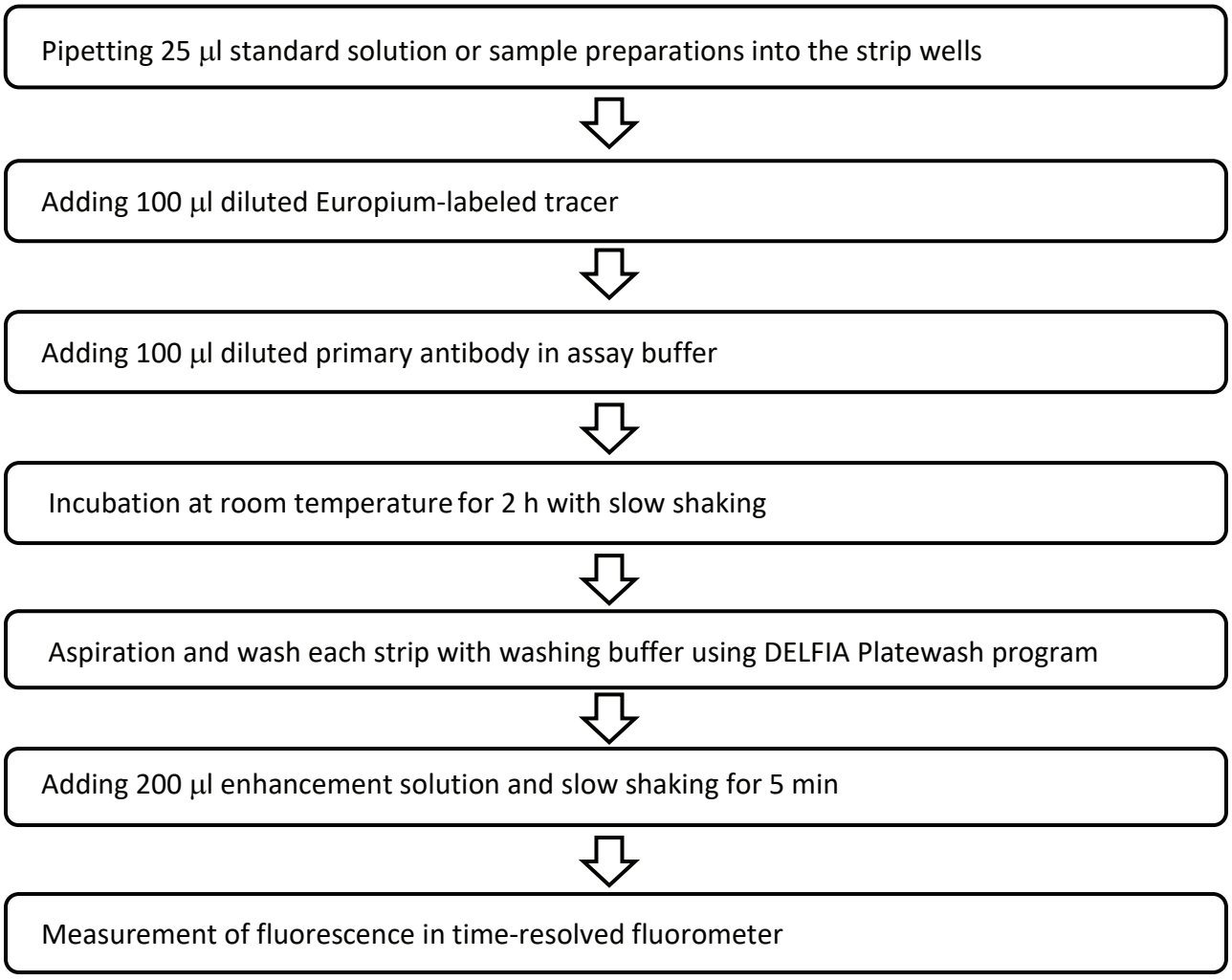


Fig. 2.9 Procedure of fluoroimmunoassay for progesterone and testosterone

Chapter 3

Establishment of a novel radioimmunoassay system for total thyroxine (T4) measurement in horse

3.1 Background

The thyroid gland synthesizes and secretes the thyroid hormones 3, 3', 5, 5'-tetraiodothyronine (or thyroxine; T4) and 3, 3', 5-triiodothyronine (T3), which play important roles in thermogenesis, growth, and metabolism in animals (Breuhaus 2006; Chen and Riley 1981; Bird et al. 1998; Todini et al. 2015). T4 is transformed to T3, the active form, by monodeiodination in peripheral tissues (Reed et al. 2004). Although over 99% of thyroid hormones circulating in blood are bound to plasma proteins, only the free forms, fT3 and fT4, active in binding thyroid hormone receptors (Chopra et al. 1975; Evinger and Nelson 1984; Messer 1993; Reed et al. 2004).

Several techniques have been routinely used to evaluate thyroid hormone concentrations in various samples (*e.g.*, plasma, serum, saliva, feces) for research and the diagnosis of thyroidal or non-thyroidal illness in humans and animals. Radioimmunoassay (RIA) has been considered to be the gold standard method, exhibiting high sensitivity and specificity, and low detection limits (Breuhaus et al. 2006; Fitzgerald and Davison 1998; Graves et al. 2006; Huszenicza et al. 2000; Kadunc et al. 2003; Slebodzinski et al. 1998; Sojka et al. 1993). Furthermore, equilibrium dialysis (Breuhaus et al. 2006), ultrafiltration (Sophianopoulos et al. 1980), enzyme immunoassay (EIA) (Brinkman et al. 2006; Medica et al. 2001; Todini et al. 2010; Todini et al. 2015), chemiluminescence immunoassay (CLIA) (Eshratkhah et al. 2011; Singh et al. 1997), chemiluminescent enzyme immunoassay (CLEIA)

(Esquivel and Ramírez 2016), and electrochemiluminescence immunoassay (ECLIA) (Eshratkhah et al. 2011; Wheeler 2013) are available for the evaluation of thyroid function in many species. Nonetheless, reference values and intervals of thyroid hormone concentrations substantially vary among measurement techniques and laboratories.

Recently, various commercial immunoassay kits that were developed for human samples are being widely applied to determine T4 and T3 concentrations for biomedical research and clinical hospital practice in animals, including horses, due to the simplicity of measurement procedure. Thyroid hormones are chemically common in human and horse. However, thyroid hormone binding proteins such as thyroid hormone-binding globulin (TBG), albumin, and thyroxin-binding prealbumin (TBPA, transthyretin) are different between species in their quantity and binding affinity (Engelking 2002). Although circulating concentrations of total T4 are the combination of T4 bound to the binding proteins and a small free portion (about 0.05% of total T4), importantly, determination of serum fT4 levels in horses does not provide any information regarding thyroid gland function that is additional to that of total T4 measurement (Sojka et al 1993). In addition, species difference in binding proteins can affect accurate detection of T4 by the kits that have been produced specifically for humans. Therefore, assay protocol optimized for total T4 of horses including dissociation of T4 from binding proteins is necessary to determine true value of circulating T4 in horses.

The aims of this study were: i) to establish a reliable and sensitive RI assay protocol, ii) to develop a method for binding protein separation that is simple and more convenient for equine T4 measurements, and iii) to evaluate the assay by comparing total T4 concentrations in horses among different chronologic ages and geographic climates.

3.2 Materials and methods

3.2.1 Animals

Twenty one Thoroughbred-trained yearling and 2 adult horses (in estrus or diestrus) were used in this study. The yearlings were raised on Hokkaido (in the temperate north) and Miyazaki (in the subtropical south) in Japan with and without artificial light supplementation from October to April. Blood samples were collected from the jugular veins into non-anticoagulant and heparinized vacutainers. For different climate comparison, blood sample collection were performed one week before light supplementation (mid-December) and then once a month in late January, late February, mid-March and early April. After centrifugation, sera and plasma were harvested and stored at -20°C until assayed. To compare the two methods in binding protein separation, and differences between yearlings and adults, we used plasma samples from Hokkaido yearlings under light supplementation. Sera collected from adult horses in their estrous and diestrous periods were used for testing in sodium salicylate method. Plasma from Hokkaido and sera from Miyazaki yearlings under natural light conditions were also employed for different climate comparisons.

3.2.2 Separation of binding proteins

The separations of binding protein in this study were categorized to 3 methods as follows:

Sodium salicylate method. The use of sodium salicylate for binding protein separation had been described previously in thyroxine evaluation (Tohei et al. 1997). A glycine-gelatin buffer (GGB) containing 2% sodium salicylate (w/v) was typically used as T4 assay buffer to dilute with standard and samples. In the present study, the original GGB was used in preparation of standard and sample solutions. Then, GGB which was modified from Tohei *et*

al. by adjusting gelatin to 0.1% (w/v); with sodium azide 0.1% (w/v) added, pH titrated to 7.4, and without sodium salicylate, was required to mix with standard and sample solutions for RIA. In addition, to investigate the effect of sodium salicylate on the binding of T4 radioligand to the first antibody, serial dilution of the original GGB were performed with modified GGB in range from 1% to 0.002% sodium salicylate containing and then were assayed.

Acid ethanol cryo-precipitation. The following extraction method was modified from the original method which was commonly performed before IGF-1 measurement (Daughaday et al. 1980). The protocol for acid ethanol method has been demonstrated in Chapter 2 (see Fig. 2.4). Standards and samples (100 μl) with the acid ethanol mixture (400 μl), which was prepared with 2 M HCl and 99.5% ethanol in a ratio of 12.5%:87.5% (v/v), were mixed in 12x75 mm glass tubes and incubated at room temperature for 30 min. After centrifugation at 4°C, 2100 g for 30 min, the supernatant was collected by decantation and neutralized with 0.855 M Tris (hydroxymethyl) aminomethane (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) solution at a ratio of 5:2, mixed thoroughly, and then stored at -20°C for 1 hr. After storage, all tubes were immediately centrifuged at 4°C, 2100 g for 30 min. The supernatants were collected into fresh tubes and diluted with gelatin-PBS which was composed of 0.05 M PBS containing 0.1 % sodium azide (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.1% gelatin and 0.1% Triton X-100 (polyoxyethylene octylphenylether), pH 7.4 (Sigma-Aldrich Co. LLC., USA) to achieve at final solution; and then stored at -20°C until assayed.

Sodium acetate ethanol method. This technique is commonly used for protein precipitation using alcohol and sodium acetate (Nomura et al. 2003). The standard and

samples (100 μl) were transferred to glass tubes. Sodium acetate ethanol mixture (300 μl) which was a mixture of 2 M sodium acetate (Wako Pure Chemical Industries, Ltd.) and 99.9% ethanol in a ratio of 5%:95% (v/v) was added to each tube, carefully mixed, and then centrifuged at 4°C, 2100 g for 30 min. The supernatant was harvested and mixed with gelatin-PBS Triton X as the final solution. The extracted standards and samples were kept at -20°C until analysis. The protocol for sodium acetate ethanol method is shown in Fig. 3.1.

Herein, the final extracted solution for both methods (the supernatant diluted with gelatin-PBS), needed to be calculated and adjusted to 50 μl/tube for RIA, which was typically in the middle of the standard curve. In addition, T4 concentrations in yearlings during growth and development were relatively high, so that a further ten-fold dilution of the final solution with modified GGB without sodium salicylate was required.

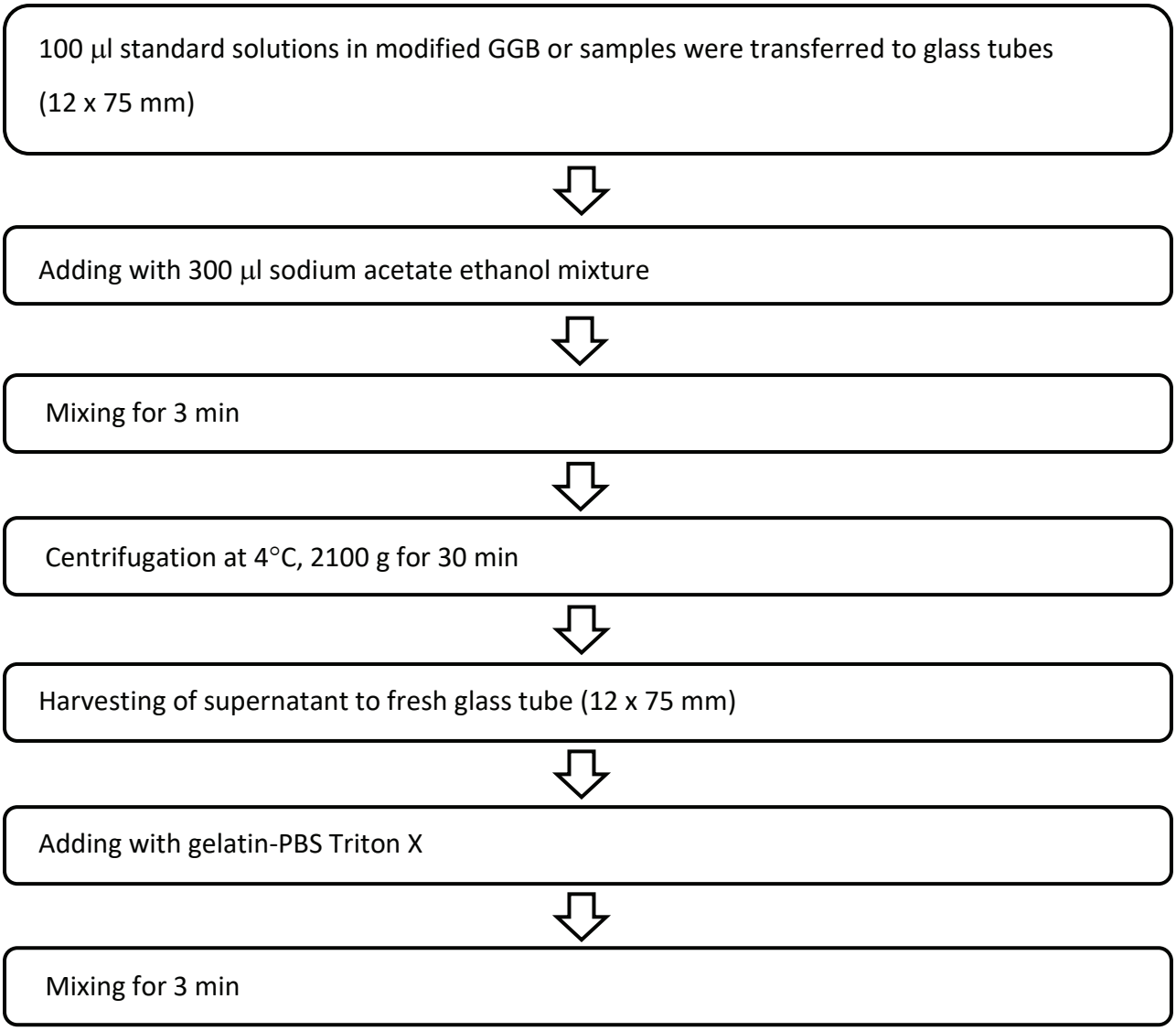


Fig. 3.1 Procedure of sodium acetate ethanol method for binding protein separation

3.2.3 Radioimmunoassay of T4

In radioiodination, tracer was prepared from thyroxine-BSA conjugate (Cat. No. 8960, Bio-Rad AbD Serotec Limited, Raleigh, NC, USA) labeled with ^{125}I (NEZ033A, PerkinElmer, Inc., Waltham, MA, USA) by the chloramine T method as the previously described (Hasegawa et al. 1986). Total thyroxine (T4) concentrations were determined by a double-antibody RIA system. All samples and standard (L-thyroxine, T2376, Sigma-Aldrich Co. LLC.) doses were measured in duplicate and triplicate in the same assay. The diluted (1:1000) primary polyclonal antibody (lyophilized rabbit anti-thyroxine BSA serum, Cat No. 65850, MP Biomedicals, LLC., Santa Ana, CA, USA) in modified GGB without sodium salicylate containing 0.4% normal rabbit serum (NRS) was added (50 μl/tube) to disposable polypropylene tubes (1.2 ml microtiter tubes for RIA, Thermo Scientific, San Diego, CA, USA). After mixing, all tubes were incubated at 4°C for 24 h. Tracer T4 labeled with ^{125}I in modified GGB without sodium salicylate (50 μl; approximately 5000 counts per minute) was added and briefly mixed. After incubation at 4°C for 24 h, the diluted (1:40) secondary antibody (anti-rabbit-gamma-globulin (ARGG), Veterinary Physiology Laboratory, Tokyo University of Agriculture and Technology) in modified GGB without sodium salicylate containing 7% polyethylene glycol (Wako Pure Chemical Industries, Ltd.) was added (50 μl/tube) to the tubes. After mixing, the tubes were then incubated at 4°C for 24 h. Thereafter, bound and free ligands were separated by centrifugation at 1700 g for 30 min, at 4°C. The supernatant was decanted and the precipitate was counted in a gamma counter (Cobra Quantum, PerkinElmer, Inc.) for 1 min. The protocol for sodium salicylate method is shown in Fig. 3.2.

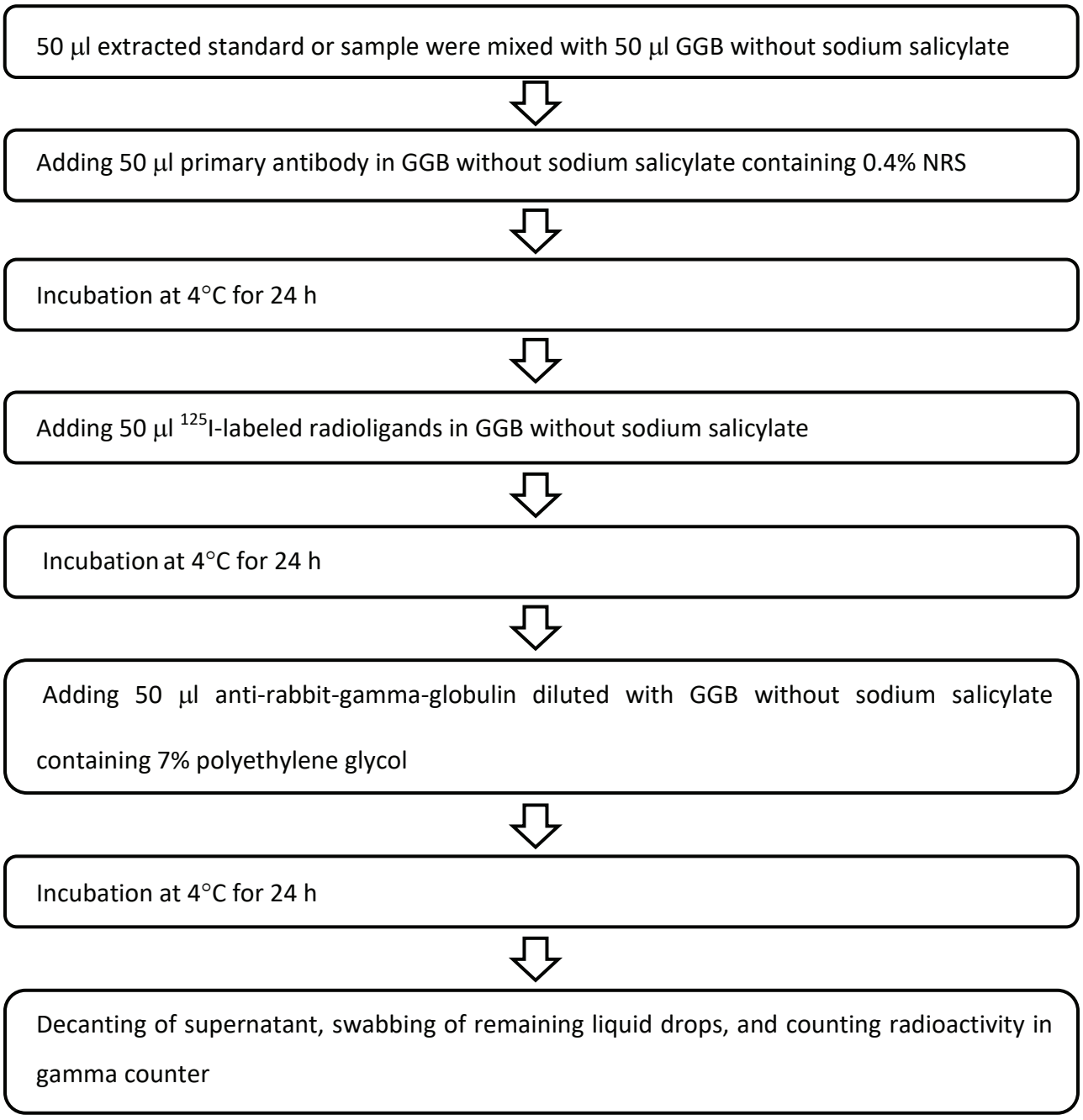


Fig. 3.2 Procedure of radioimmunoassay for T4

3.2.4 Statistical analysis

All results are expressed as means ± standard errors of the means (SEM). Linear regression was applied to logarithmically transformed dose concentrations for the standard curve. The differences in means for T4 values between different binding-protein separation methods and age groups were analyzed using an unpaired Student's t-test with Graph Pad Prism, ver. 5. For different climates in the longitudinal study, the repeated-measures data were analyzed using the generalized least squares (GLS) (Gueorguieva and Krystal 2004; Ugrinowitsch et al. 2004). Bonferroni's multiple comparisons were used to determine the differences in means of T4 values using R software. The level of significance was set at 0.05.

3.3 Results

Standard curve with sodium acetate ethanol methods

The representative dose-response curve for T4 demonstrated linearity between logarithmic dose 0.0078 and 1 ng/tube. The dose-response curve for horse samples at each age paralleled the standard curve (Fig. 3.3)

Comparison of acid ethanol and sodium acetate ethanol methods with respect to circulating T4 concentrations

The circulating T4 concentrations of two different separation methods were analyzed in triplicate in the same assay. After extraction, two doses of the extracted samples from both methods were selected, at 4.4 μl/tube for the higher concentration and 0.44 μl/tube for the lower concentration. The lower dose of 0.44 μl/tube was appropriate and closer to 50% binding on the standard curve. The mean concentration of T4 in the 0.44 μl/tube was not significantly different between acid ethanol and sodium acetate ethanol methods

(*P*=0.438) (Table. 3.1). Also, slopes of both linear regression lines were not significantly different (*P*=0.614) (Fig. 3.4). The intra-assay coefficient of variance was 5.0%.

RIA with sodium salicylate methods

As a fundamental method for binding protein separation using 2% sodium salicylate in original GGB, standards and sera from adult horses were prepared by mixing with the original GGB to obtain expected dose and were then diluted with modified GGB (without sodium salicylate) to 0.29% and 0.57% sodium salicylate, respectively, for the assay. The representative dose-response curve for T4 showed linearity between the logarithmic dose 0.0195 and 10 ng/tube (Fig. 3.5). The dose- response curves for adult sera (from horses in estrus or diestrus) did not parallel the standard curves. In fact, the percent bound for both estrous and diestrous sera were quite low at every dose, and also lower than for acid ethanol and sodium acetate ethanol methods, when comparing a similar dose (Fig. 3.5). Furthermore, the serial dilution of sodium salicylate containing in the original GGB using in the assay showed that sodium salicylate affected percent bound in dose dependent of manner. However, the percent bound seemed to be quite constant in range from 0.016% to 0.002% of sodium salicylate (Fig. 3.6). In addition, the use of 0.016% sodium salicylate for RIA still presented very low levels of circulating T4 in horse sample (data not shown).

Comparison of circulating T4 concentrations between yearling and adult horses with respect to the sodium acetate ethanol method

To evaluate whether the novel RIA system in the present study would provide reliable measurements of T4 in horse samples, we determined circulating T4 concentrations after sodium acetate ethanol extraction between different ages of horses (yearlings and adults), in triplicate in the same assay. Yearlings showed a significantly higher mean T4

concentration compared to adults at a dose of 0.44 μl/tube (*P*=0.013) (Table 3.2). The mean concentration of T4 in yearlings was 6 times as high as in adult horses. Using linear regression analysis, both groups exhibited dose dependency and parallelism, with slopes that were not different (*P*=0.884) (Fig. 3.3). The intra-assay coefficient of variance was 8.2%.

Comparison of circulating T4 concentrations between Hokkaido and Miyazaki yearlings with respect to the sodium acetate ethanol method

To investigate whether the RIA was an appropriate tool for determination the true value of T4 in horse. Plasma and sera were obtained from growing horses raised in different climatic areas. Hokkaido, in the temperate north, and Miyazaki, in the subtropical south of Japan were selected for this experiment. Samples were then determined in duplicate after sodium acetate ethanol extraction in the same assay. There were no significant differences in circulating T4 concentrations between Hokkaido and Miyazaki male yearlings throughout the experiment (*P*>0.05). In fact, female Miyazaki yearlings showed significantly lower T4 levels compared with those of Hokkaido only at late January (*P*=0.007). However, circulating T4 concentrations in both genders of Hokkaido yearlings tended to be greater than those in Miyazaki throughout these periods (Fig. 3.7A and 3.7B). The levels of circulating T4 were also not significantly different among time periods in the Hokkaido and Miyazaki yearlings during the seasonal transition from winter to early spring (*P*>0.05).

3.4 Discussion

Most of circulating thyroid hormones are bound to plasma proteins. The free forms are small portion in blood. There are differences of T4 binding proteins between species in their quantity and binding affinity (Engelking 2002). These can affect accuracy of detection in

T4 concentration. Thereupon, it is essential to remove any binding proteins in serum or plasma before performing immunoassays. In the present study, we adapted two extraction techniques, acid ethanol and sodium acetate ethanol, and evaluated their suitability for determining equine T4 concentrations. The acid ethanol method, which has been commonly used for insulin-like growth factor I (IGF-1) measurement (Daughaday et al. 1980), requires proceeding in several steps that increase protocol time compared with the sodium acetate ethanol method. Therefore, we examined and compared the efficiency of protein extraction between both modified methods. The present study revealed that both extraction procedures had the capability to separate bound proteins from T4, appropriately showing parallelism to dose-response curve for extracted T4 standards. No differences in T4 concentrations between the two techniques indicated that use of the modified sodium acetate ethanol method not only possessed effectiveness in protein extraction similar to that of acid ethanol, but was also simpler, especially in case of large sample quantities.

In the present study, GGB was modified by exclusion of sodium salicylate, which has been used as an inhibitor or displacer of thyroid hormone binding to serum protein in thyroid hormone measurements (Tohei et al. 1997). In previous publications, sodium salicylate was capable of binding plasma proteins in humans and other species (McArthur and Smith 1968; Sturman and Smith 1967; Wang et al. 1999), and the application of 2% sodium salicylate was required to separate binding proteins in serum samples. However, our study found that 2% sodium salicylate markedly affected the binding of T4 radioligand to the first antibody. To avoid this effect on binding, I tried to find an appropriate percentage of sodium salicylate which still existed with ability in binding protein separation but had no effect on tracer-antibody binding. This trial found that sodium salicylate should be low as 0.01% (Fig. 3.6). Consequently, the dilution to 0.01% made T4 levels too low to detect in

horse serum samples. For this reason, I used the alternative sodium acetate ethanol method instead of sodium salicylate for binding protein separation prior to RIA.

To validate our assay, I investigated parallelism among dose-response curves of yearlings, adults, and T4 standards (Fig. 3.3). As a result, assayed T4 concentrations in yearlings were clearly 6-fold higher than in adult horses, consistent with previous studies showing that young horses had T4 higher than adults (Blackmore and Brobst 1981; Gupta et al. 2002). It is reasonable that yearling horses in the process of development manifest higher metabolism than adults. In addition, we investigated and compared circulating T4 levels in different climatic conditions in yearling horses. Hokkaido in the northern part of Japan is located in a temperate zone such that it had lower temperatures throughout the experimental periods compared with the Miyazaki setting in the subtropical south. In this experiment, there was a tendency for circulating T4 concentrations to be higher in Hokkaido yearlings than in Miyazaki yearlings throughout these periods. This result is consistent with previous research in which horses staying in colder condition tended to have T4 levels higher than horses in warmer climates during the winter (Fazio et al. 2012; Mejdell and Bøe 2005). Our assay was similarly likely to have a sensitivity and specificity that was sufficient for the evaluation of T4 concentrations of physiologic responses at different ages and in different climates.

I believe that total T4 determinations are as important as fT4 for analyses of thyroid gland function, and this is supported by previous reports that fT4 concentrations in plasma were highly correlated with total T4 (Brinkmann et al. 2016; Elliott et al. 2013; Welcker et al. 2013). The value in adult horses in the present study was similar to reference values in several publications using various measurement methods, which reported that total T4 in adult equines were 11-36 ng/ml by CLEIA (Medica et al. 2011), 17-31 ng/ml (Mejdell and Bøe

2005); 25.2 ± 11 (winter) and 10.4 ± 6.3 (summer) ng/ml (Brinkmann et al. 2016) by CLIA; 30.72 ± 6.47 ng/ml (Medica et al. 2011); 40.3 ± 2.26 ng/ml (Todini et al. 2010); 24.1-49.47 ng/ml (Fazio et al. 2012); 26.95 ± 1.35 ng/ml (Gupta et al. 2002) by ELISA; 18-30 ng/ml (McBride et al. 1985); 6.2-25.1 ng/ml (Sojka et al. 1993) by RIA; and 4.7-35.74 ng/ml by equilibrium dialysis with RIA (Breuhaus 2006). Interestingly, total T4 levels in yearlings obtained from our RIA, 209.17 ± 15.05 ng/ml, were dramatically different from the reference value of 35 ng/ml (Reed et al. 2004), 68.68 ± 2.0 ng/ml in horses at 6 months to 1 year of age, or from 34.61 ± 0.92 ng/ml in 1-3 year old horses (Gupta et al. 2002). This high T4 levels in yearling horses probably derived from my removal of binding protein using the sodium acetate ethanol method when compared to other techniques (e.g., ELISA and RIA kits) in previous reports (Gupta et al. 2002 and Reed et al. 2004). In conjunction of the present study with previous reports showing that the similarity in adults and difference in yearlings of T4 values might indicate that our immunoassay, compared with other methods, appears to be more physiological and appropriate in revealing the true value of total T4 in equine species.

In conclusion, this is the first report describing a modified sodium acetate ethanol technique that is simpler and more convenient for bound T4 protein extraction, and the first study in which a reasonable radioimmunoassay was established specifically for circulating total T4 measurements in horses. Other physiologic changes in T4 be should be investigated in order to more fully understand thyroid gland function in yearling horses.

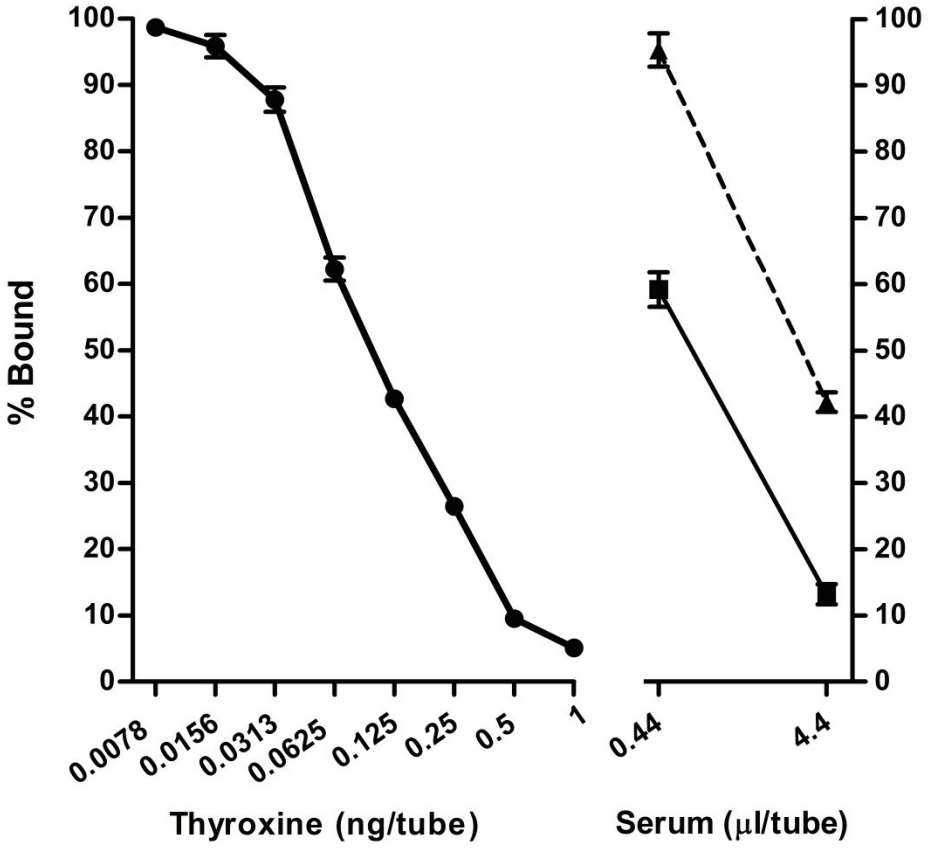


Fig. 3.3 Radioimmunoassay dose-response curves for total T4 concentrations with sodium acetate ethanol extraction. T4 standards (●) from 0.0078 to 1 ng/tube, yearling plasma (■) and adult sera (▲) using ^{125}I-labelled thyroxine as tracer. The X axis shows doses of T4 on a logarithmic scale. Each value is expressed as the mean ± SEM of triplicate measurements. Comparison of percent bound was obtained from yearling plasma and adult sera, and showed that the regressed line paralleled the T4 standard (y= -13.89x ± 74.30 and -13.42x ± 101.2, r^2=0.878 and 0.993; *P*<0.05 and *P*<0.001, respectively).

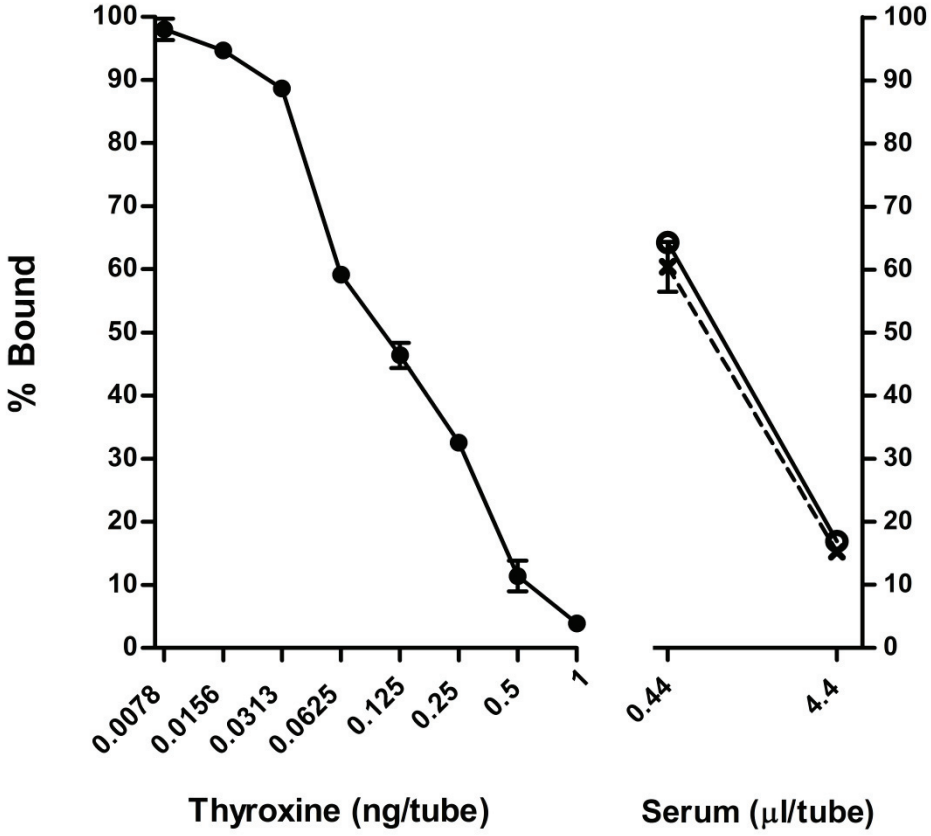


Fig. 3.4 Radioimmunoassay dose-response curves of total T4 concentrations in yearling plasma. T4 standards (●) from 0.0078 to 1 ng/tube were extracted by acid ethanol method. Samples were extracted by sodium acetate ethanol (O) and acid ethanol (✕) methods. Each value is expressed as the mean ± SEM in triplicate measurements. Both methods of extraction, sodium acetate ethanol and acid ethanol, showed similar regression lines (y= -11.96x ± 69.55 and -11.41x ± 65.46, r^2=0.996 and 0.970; *P*<0.0001 and *P*<0.001, respectively).

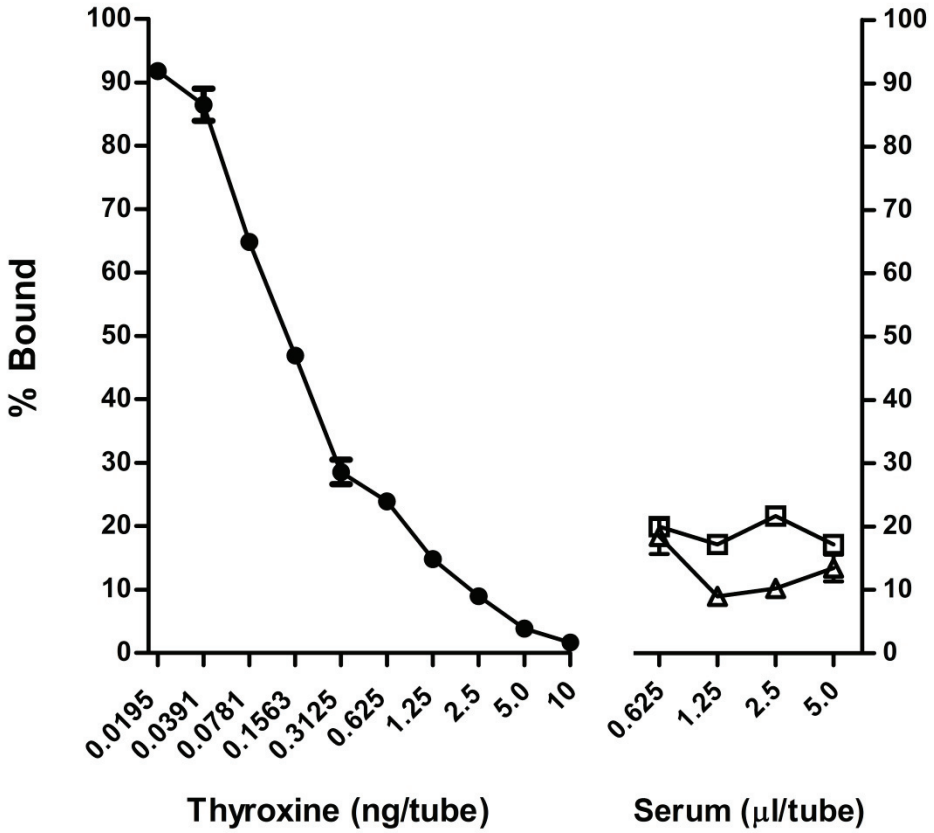


Fig. 3.5 Radioimmunoassay dose-response curves of total T4 concentrations in adult sera using sodium salicylate for extraction. Estrus (□) and diestrus (△) periods were extracted *via* the method of bound protein separation with sodium salicylate. Each value is expressed as the mean ± SEM in triplicate and duplicate measurements for standard and serum samples.

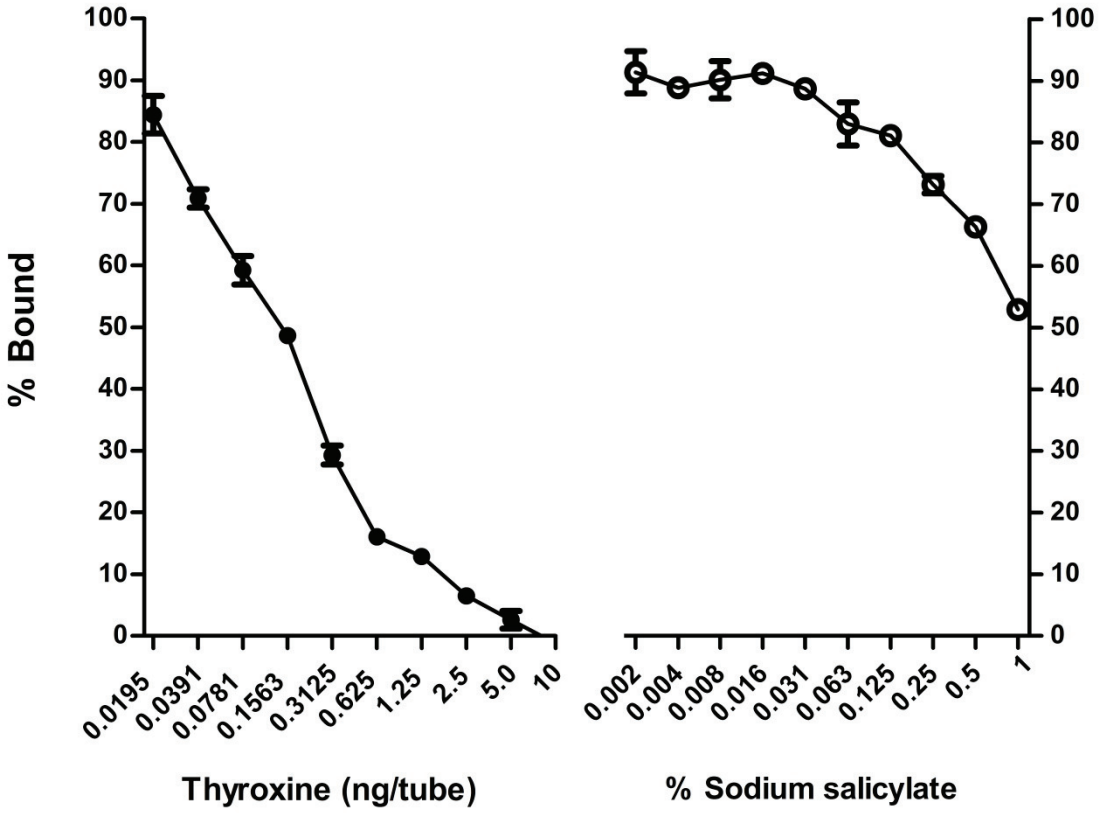


Fig. 3.6 Radioimmunoassay dose-response curves of total T4 concentrations using sodium salicylate for binding protein separation. T4 standards (●) from 0.0195 to 10 ng/tube and glycine gelatin buffer containing with sodium salicylate (○) from 1% to 0.002% used ^{125}I-labelled thyroxine as tracer. Each value is expressed as the mean ± SEM in triplicate measurements.

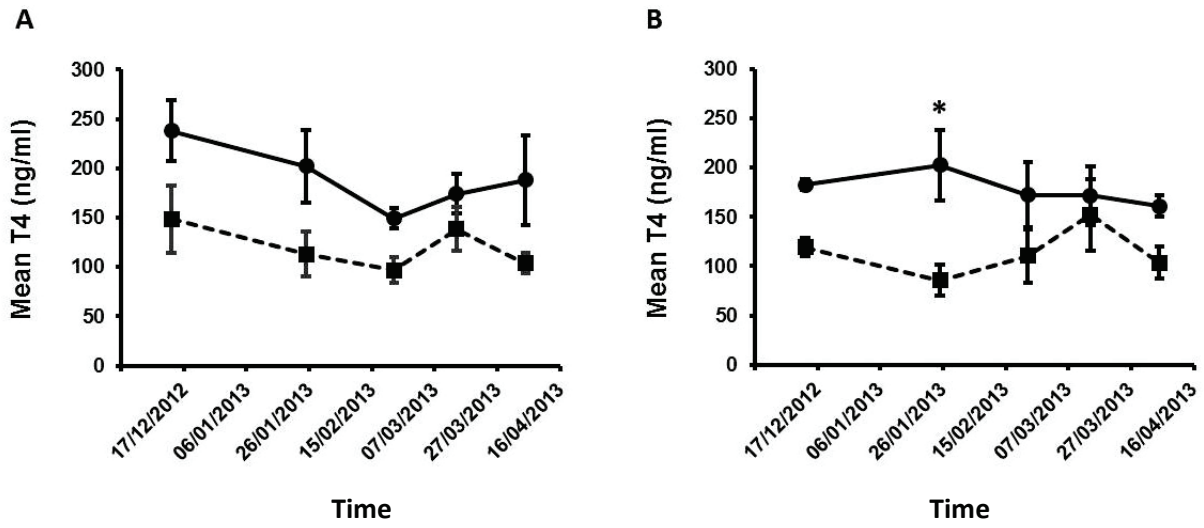


Fig. 3.7 Comparison of mean total T4 concentration between Hokkaido (●, N = 10) and Miyazaki (■, N = 10) yearlings, both colts (A) and fillies (B) from December to April. Each value is expressed as means ± SEM. * Indicates that the T4 levels differ significantly (P<0.05) between horses in the two climate groups in each period and for each sex.

Table 3.1 Comparison of serum total T4 concentration in yearling horses between two different extraction methods

Dose	Mean total T4 concentration in horse serum (ng/ml)	
(μl/tube)	Sodium acetate ethanol	Acid ethanol
0.44	174.37 ± 3.33	197.32 ± 22.27

Data were expressed by mean ± SEM (P>0.05).

Table 3.2 Comparison of serum total T4 concentration between yearling and adult horses

Dose	Mean total T4 concentration in horse serum (ng/ml)	
(μl/tube)	Yearlings	Adults
0.44	209.17 ± 15.05[a]	34.92 ± 13.69[b]

Data were expressed by mean ± SEM at doses of 0.44 μl/tube.

[a, b] Denotes significant difference P<0.05 between different age groups.

Chapter 4

Comparison of metabolic and reproductive endocrine functions and body physical growth between north and south climates of Japan in Thoroughbred trained yearling horses under natural condition

4.1 Background

Horse is expected to have not only good performances but also sound body physical conditions for achievement in racing industry including the fertility in breeding. Normally, horse is long-day seasonal breeder with highest reproductive activity in spring to summer but lowest in autumn to winter. Japan, long island lies in both northern and eastern hemispheres, has large difference in latitude between the northern and the southern part leading to two different climates; temperate and subtropical, respectively. According to weather report of Japan Meteorological Agency in 2012-2014, Hidaka area of Hokkaido in the north had lower temperature than Miyazaki in the south throughout those two year seasons especially in late autumn to winter and the large difference of mean daily minimal temperature was found between the two places (Fig. 4.1). Also, sunshine duration in Hokkaido shorter than Miyazaki almost of years and much lower during autumn and winter season (Fig. 4.2). During decades, Hokkaido has been known as the main land for Thoroughbred horse breeding. Most of Thoroughbred race horses have been produced in Hokkaido prefecture. The climate distinctions between the north and the south of Japan incidentally consistent to some observation that horse reared in the southern area grow well and seemed to be bigger than horses in the north when compared in the second spring of

horse's life. Therefore, the difference of climatic circumstance may influence on growth and reproductive function in horses which rear between the north and the south of Japan.

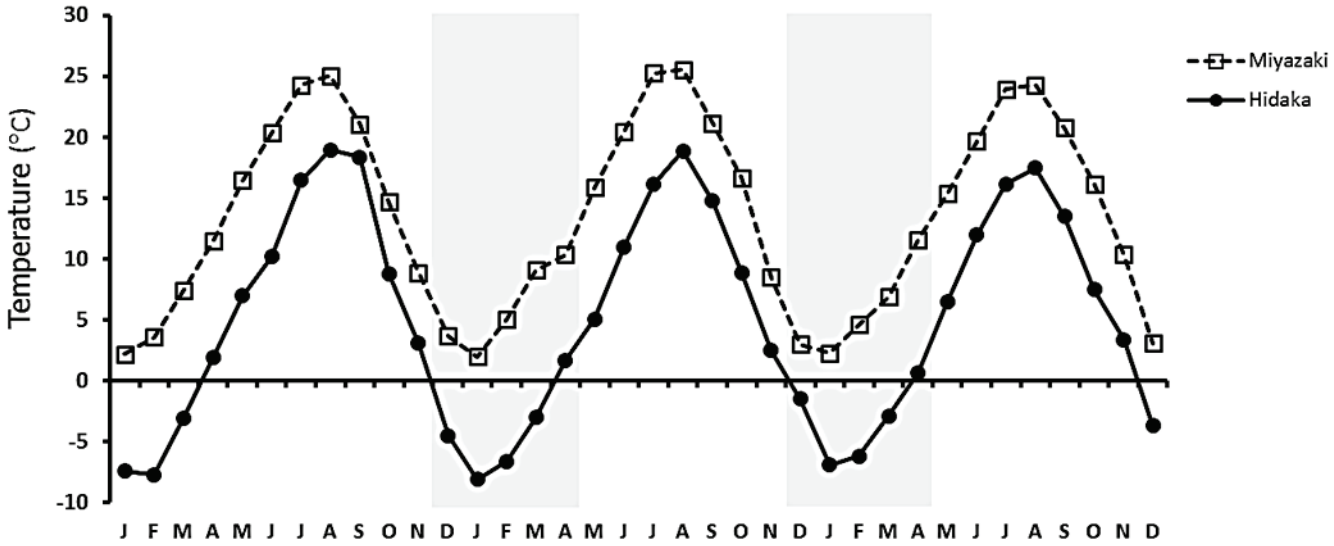


Fig. 4.1 Mean daily minimum temperature between Hidaka area (Urakawa, Hokkaido) and Miyazaki during 2012-2014. (Source from Japan Meteorological Agency)

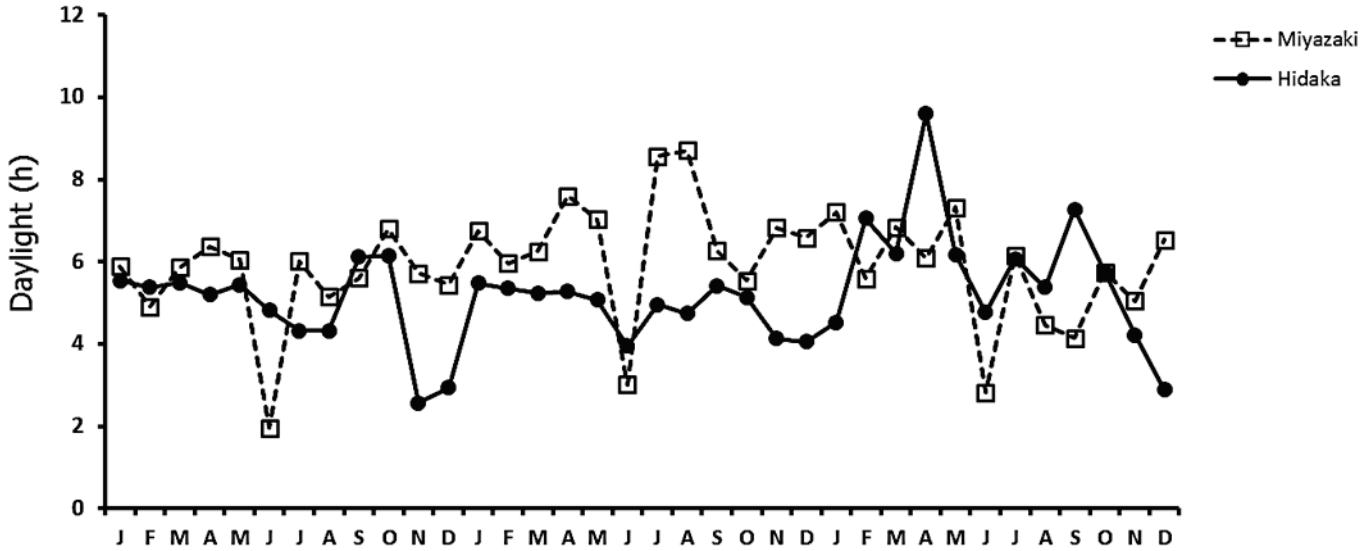


Fig. 4.2 Monthly total of sunshine duration between Hidaka area (Urakawa, Hokkaido) and Miyazaki during 2012-2014. (Source from Japan Meteorological Agency)

Hormones involve in metabolism and development such as thyroid and insulin-like growth factor I (IGF-1). Thyroid hormones, thyroxine (T4) and tri-iodothyronine (T3), plays important roles in thermogenesis, metabolism, and growth in both human and animals (Breuhaus et al. 2006; Chen and Riley 1981; McGuire et al. 1991, Bird et al. 1998). Moreover, thyroid hormones are able to be an indicator of metabolic rate in resting stage under various environmental and climatic conditions (Fröhli and Blum 1988; Melesse et al. 2011; Nilsson et al. 1985; Welcker et al. 2013). The increase of T4 and T3 concentrations in horses were derived from cold exposure (McBride et al. 1985). Previous researches reported that T4 and T3 increased the metabolism of the most cells and stimulate growth in young animals (Latimer et al. 2003; Thrall et al. 2004; Chatterjea and Shinde 2005). IGF-1 acts as endocrine and autocrine/paracrine factor in local tissue of various species (Brokaw et al. 2007; Roser 2008; Colon et al. 2007; Gnessi et al. 1997; Robaire and Hamzeh 2011; Yoon and Roser 2010; Yoon et al. 2011; Yoon and Roser 2011). It has involves to steroidogenesis (Lin et al. 1986a,b; Kasson and Hsueh 1987; Gelber et al. 1992; Wang and Hardy 2004; Bernier et al. 1986; Perrard-Sapori et al. 1987) and promote body growth in animals (Doherty et al. 1994; Herrler et al. 1998; Fabian et al. 2004; Wang et al. 2009). Regarding the reproduction, prolactin (PRL) is greater in long day season in horses (Gerlach and Aurich 2000; Curlewis 1992). It had been known as the main hormone which is related to lactation, reproduction, and also responsible for hair shedding (Doherty et al. 1994; Herrler et al. 1998; Fabian et al. 2004; Wang et al. 2009). Moreover, PRL had paracrine/autocrine functions in follicle and corpus luteum (Daoud and Ezzo 2014) and was involved in stimulation of LH receptor expression for testosterone secretion (Hair et al. 2002). Interestingly, some connection among thyroid hormone, prolactin and reproductive hormone has been studied (Evans et al. 1991; Kunii et al. 2015; Suzuki et al. 2015), but the definite mechanism is still unclear.

Therefore, the objectives of this study were to 1) determine changes in metabolic and reproductive hormones and body physical growth in Thoroughbred trained yearling horses and 2) compare those changes between Hokkaido and Miyazaki horses under natural condition.

4.2 Materials and methods

4.2.1 Animals

During two year seasons (2012-2013 and 2013-2014), total 47 Thoroughbred trained yearling horse from 1 year old of age at September in autumn to less than 2 year of age at April in spring which were reared in 2 facilities of the JRA, Hidaka Training and Research Center in Hokkaido (temperate north, latitude 42.2° and longitude 142.8°) or Miyazaki Training Yearling Farm in Miyazaki (subtropical south, latitude 31.9° and longitude 131.4°), were chosen to be subjects in this study. Twenty-five control yearlings (12 colts, 13 fillies) in Hokkaido and 22 control yearlings (10 colts, 12 fillies) in Miyazaki were used under natural condition throughout experimental periods. (Diets and exercise program see Chapter 2)

4.2.2 Growth parameters and hair coat conditions

Body weight, body height, and girth and cannon bone circumferences were measured once a month by weight machine, height ruler, and measuring tape, respectively (Fig. 4.3). Increment percent of all parameters were calculated as follow:

$$\text{Increment percent} = \frac{[\text{Value of current month} - \text{Value of previous month}] \times 100}{\text{Value of previous month}}$$

Means of raw values and percent of increments were used for comparison between groups. In addition, hair coat condition i.e. type of feather, thickness, shining and shedding were evaluated and scored by three veterinarians in November, January and April in each year for two year seasons. The scoring system was categorized to; 1=poor, 2=normal, and 3=excellent. Mean scores were compared between groups.

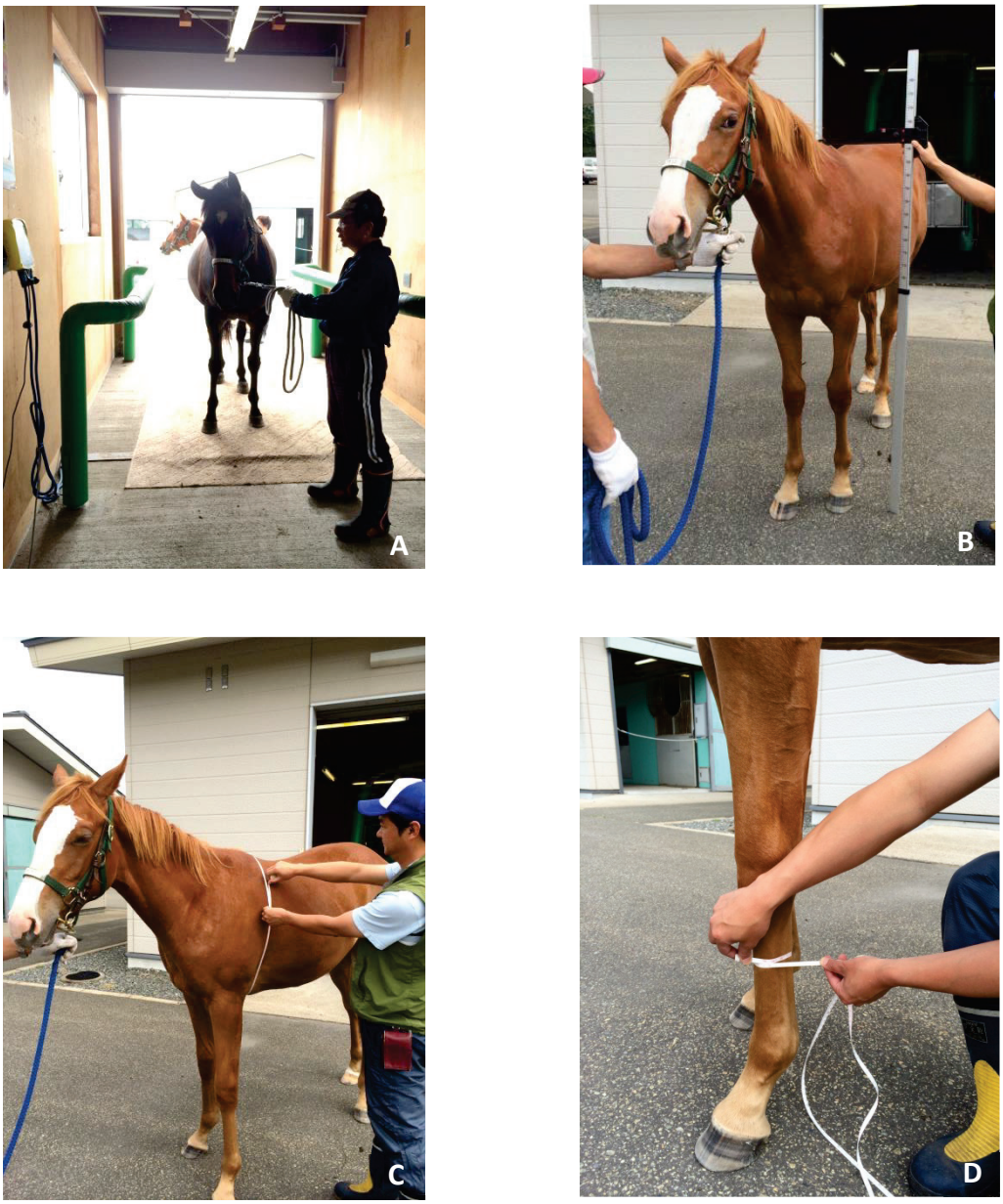


Fig. 4.3 Measurements of body physical growth; (A) body weight; (B) body height (C) girth; and (D) cannon bone circumferences.

4.2.3 Endocrine parameters

Blood samples were collected from jugular veins into non-anticoagulant or heparinized vacutainers between 9:00 and 12:00 hr one week before light supplementation (mid-December) then once a month from January to April (late January, late February, mid-March and early April) in each year for two year season. After centrifugation, sera of Miyazaki and plasma of Hokkaido were harvested and stored at -20°C until assayed. For parameter of endocrine function, circulating total T4 (using modified sodium acetate ethanol method for binding protein separation), IGF-1, PRL, immunoreactive (ir-) inhibin and cortisol levels were measured by radioimmunoassay system. The intra- and inter-assay coefficients of variation were 9.7% and 5.9% for T4, 8.6% and 5.0% for IGF-1, 10.0% and 5.3% for PRL, 9.6% and 18.0% for ir-inhibin and 9.6% and 1.3% for cortisol, respectively. For measurement of circulating sex steroid hormones, estradiol-17β and progesterone in fillies, and testosterone in colts were performed by time-resolved fluoroimmunoassay (DELFIA, Eu-Labelling kit, PerkinElmer, USA). The intra- and inter-assay coefficients of variation were 5.8% and 4.4% for estradiol-17β, 4.6% and 4.9% for progesterone and 3.8% and 6.7% for testosterone, respectively. The measured values of hormones were not different between sera and plasma samples.

4.2.4 Determination of ovarian and testicular function

First expected activity of ovary in fillies and testis in colts were arbitrarily defined when the circulating concentrations of progesterone reached over 1 ng/ml and testosterone reached 0.5 ng/ml or higher, respectively.

4.2.5 Statistical analysis

All statistical analysis was performed using R software. The results were defined as mean ± standard errors of the means (SEM). The repeated measures data in longitudinal sampling were analyzed using generalized least squares (GLS). Differences in means of all parameters between Hokkaido and Miyazaki groups under natural condition and among time study were adjusted by Bonferroni's multiple comparison tests. The significance level was set at alpha = 0.05.

4.3 Results

Growth parameters

The monthly means of body weight, body height, girth and cannon bone circumferences comparing between Hokkaido and Miyazaki horses are shown in Fig. 4.4A-D. There were no significant differences in the means of body weight (Fig. 4.4A), height (Fig. 4.4B) and girth circumference (Fig. 4.4C) between Hokkaido and Miyazaki horses, however Hokkaido colts and fillies seemed to have those three values lower than Miyazaki horses. Cannon bone circumferences were significantly lower in Hokkaido colts and fillies when compared to the Miyazaki (Fig. 4.4D). Remarkably, increment percent of the body weight in Hokkaido colts and fillies tended to decrease dramatically in January and eventually increased in February to March, whereas the decline of body weight increment in Miyazaki horses were not notable during January (Fig.4.5A). Moreover, these trends were similarly found in the increment percent of height in fillies (Fig.4.5B; b), girth in both colts and fillies (Fig.4.5C; a-b) and cannon bone circumferences in fillies of Hokkaido (Fig.4.5D; b).

Hair coat conditions

The comparison of hair coat condition scores between Hokkaido and Miyazaki horses are demonstrated in Fig. 4.6. There were no significant difference of hair coat scores between Hokkaido and Miyazaki yearlings at November and January. In contrast, both Hokkaido colts and fillies had hair coat scores lower than the Miyazaki horses significantly at the end of experiment time (April).

Endocrine functions

Circulating total T4, IGF-1, PRL, cortisol (Fig. 4.7A-D), estradiol-17β, progesterone, testosterone, and ir-inhibin (Fig. 4.8A-D) were compared between Hokkaido and Miyazaki horses. The total T4 concentrations in Hokkaido colts and fillies tended to be higher than those of Miyazaki (Fig. 4.7A). No significant differences of T4 concentrations were found among periods. In contrast, the IGF-1 concentrations in Hokkaido colts were significantly lower than that in Miyazaki while fillies were not (Fig. 4.7B). Also, Miyazaki colts and fillies had significantly higher prolactin levels when compared to Hokkaido horses (Fig. 4.7C). The concentrations of estradiol-17β in Miyazaki fillies were higher than those of Hokkaido significantly (Fig. 4.8A). For circulating progesterone (Fig. 4.8B), testosterone (Fig. 4.8C) and ir-inhibin (Fig. 4.8D) concentrations, there were no significant differences between Hokkaido and Miyazaki groups. First expected activity of ovary and testis were found around early of April in fillies and late of February in colts, respectively. In cortisol, the levels between Hokkaido and Miyazaki groups were not different significantly, but cortisol levels in both Hokkaido colts and fillies seem to be high over the Miyazaki in late February and early April (Fig. 4.7D).

4.4 Discussion

In natural condition, the comparison between Hokkaido and Miyazaki horses on body growth, metabolic and reproductive hormonal changes revealed that Hokkaido colts and fillies were inferior to the Miyazaki yearling horses. In the present study, all growth parameters (body weight, height, girth and cannon circumferences) in Hokkaido colts and fillies tended to be lower than Miyazaki horses consistent with previous research reporting that growth rate was higher in both Miyazaki colts and fillies compared to Hokkaido yearlings (Mizukami et al. 2015). Interestingly, all growth increments declined dramatically in Hokkaido horses in January followed by the increasing in February to March eventually, whereas the decline of those increments in Miyazaki horses were not notable during January. This remarkable point indicated that Hokkaido in January, the coldest month possessing lowest minimal temperature was hard time and resulted in slow growth rate in Hokkaido horses. After acclimatizing to severe cold, the growth rate returned to increase during February to March. Contrasting to Miyazaki horses, their growth rate kept increasing and reached maximal rate in January. Then, growth rate tended to slow down to be lesser in February to March. These suggested that different weather conditions led to responding to climate for growth in different way. For hair coat condition, both Hokkaido colts and fillies had hair coat scores lower than that in Miyazaki significantly, being consistent with the lower levels of prolactin in Hokkaido colts and fillies when compared to the Miyazaki. These results were in accordance with previous study that prolactin had responsibility in hair shedding in horses (Nambo et al. 2010; Kunii et al. 2015).

Regarding total T4 in the present study, thyroxine which is synthesized and secreted by thyroid gland plays the key roles in regulating of body temperature, metabolism and growth (Bird et al. 1998; Breuhaus et al. 2006; Chen and Riley 1981). T4 levels in Hokkaido

colts and fillies tended to be higher than in Miyazaki throughout the periods under natural light. Our results indicated that horses raised in the colder north had higher basal level of thyroid hormone than horses dwelled in the milder south. Nevertheless, the higher level of T4 in Hokkaido horses did not conform to the growth profile. It can be suggested that Hokkaido horses adapted in response to lower ambient temperature for maintenance of body homeostasis in long term survival instead of growing. Moreover, the current study did not find significant differences in T4 levels among periods from winter to early spring however little fluctuations of T4 levels were shown accordantly to other findings (Fazio et al. 2012; Irvine 1967; Johnson 1986; McBride et al. 1985). These may suggest that chronic cold exposure during seasonal change did not affect T4 concentration due to capability in acclimatization of horses. Several studies about thermoregulation of horse in winter weather clarified that T4 secretion was transiently elevated during acute or short cold exposure in adult horses (Irvine, 1967; McBride et al. 1985). For chronic cold weather, serum thyroid hormone and metabolic rate did not change significantly (Mejdell and Bøe 2005). According to the limitation of cold resistance in horses, lower critical temperature (LCT), the temperature which metabolic heat production needs to increase for body core temperature maintenance (Curtis 1983) was estimated to be -11°C in yearling horses with ad lib fed (Cymbaluk 1994; Cymbaluk and Christison 1989a) while it was around 0°C in limit-fed for normal growth in yearlings (Cymbaluk 1994; Cymbaluk 1990). Typically, horses need 10-21 days to acclimatize to cold weather and require another 10-21 days for more diminished temperature (Cymbaluk 1994). When reaching LCT, physiological, metabolic and behavioral changes would occur to reduce heat loss and conserve energy (Cymbaluk 1994). Generally, in other species for long term exposure to low temperature, thyroid hormone levels were increased resulting in increasing of metabolism and heat production in sows (Anderson

2000), sheep (Sano et al. 199) and cattle (Christopherson et al. 1979) contrary to horses employing a state of hypometabolism to keep body warm (Brinkmamm et al. 2012, 2016; McBride et al. 1985).

In the present study, circulating IGF-1 concentrations in Hokkaido colts were significantly lower than Miyazaki colts consistent to previous study (Mizukami et al. 2015). IGF-1 was mainly secreted by liver and induced by growth hormone from pituitary gland. It plays a role in contribution for body growth in animals including the period of embryonic development (Doherty et al. 1994; Herrler et al. 1998; Fabian et al. 2004; Wang et al. 2009). Moreover, IGF-1 was involved in steroidogenesis in Leydig cells of various species (Lin et al. 1986a,b; Kasson and Hsueh 1987; Gelber et al. 1992; Wang and Hardy 2004; Bernier et al. 1986; Perrard-Sapori et al. 1987), and promoted follicular growth and enhanced ovarian activity in mares (Derar et al. 2005; Hammond et al. 1991, Derar et al. 2011). According to the reproductive endocrine function, the levels of progesterone in fillies and testosterone in colts of both Hokkaido and Miyazaki were low from December and then increased to reach over 1 ng/ml at early April and 0.5 ng/ml around late February, respectively. These showed that young horses seemed to have first ovarian and testicular activities in early April for fillies and late February for colts, respectively. Furthermore, circulating estradiol-17β concentration in the present study was similar to previous report (Mizukami et al. 2015) showing that the levels of estradiol-17β were lower in Hokkaido fillies compared to Miyazaki.

The current study found that the cortisol levels were not different between Hokkaido and Miyazaki horses significantly however Hokkaido horses tended to have the cortisol levels higher than Miyazaki in February and April. Previous research reported that prolactin stimulated ACTH production and cortisol secretion in rat. Some study noted that cortisol was accordant to prolactin secretion. In contrast, our study did not found any relation between

cortisol and prolactin levels. The present study could suggest that the higher cortisol levels in Hokkaido horses presumably caused from exercising program rather than the cold stress. In February, Hokkaido horses were trained with 800 m flat and slope courses while Miyazaki used only 1600 m flat track. For April, cover snow had melted so that the training program in Hokkaido horses changed to using the 1600 m flat and slope courses whereas Miyazaki still used 1600 m flat track. This indicated that exercising program in Hokkaido seemed to be harder than in Miyazaki.

From all of study results, it was clarified that body growth and development of gonadal function was slower in Hokkaido yearlings compared to Miyazaki colts and fillies. According to the weather reports from the Japan Meteorological Agency from 2012 to 2014 as the years of this experiment, the mean daily minimal temperature in Hidaka area of Hokkaido was lower than Miyazaki throughout the years (9-10°C approximately), especially in winter season around -9°C in Hokkaido whereas 2-3°C in Miyazaki. In addition, during winter period the daylight hour was about one hour shorter in Hokkaido than in Miyazaki. These climate distinction in accordance with our results suggested that the differences in climates between the north and south of Japan would affect growth and early reproductive development in young horses.

In conclusion, Hokkaido yearlings seemed to have inferiority of growth and reproductive function to the Miyazaki yearlings under natural condition.

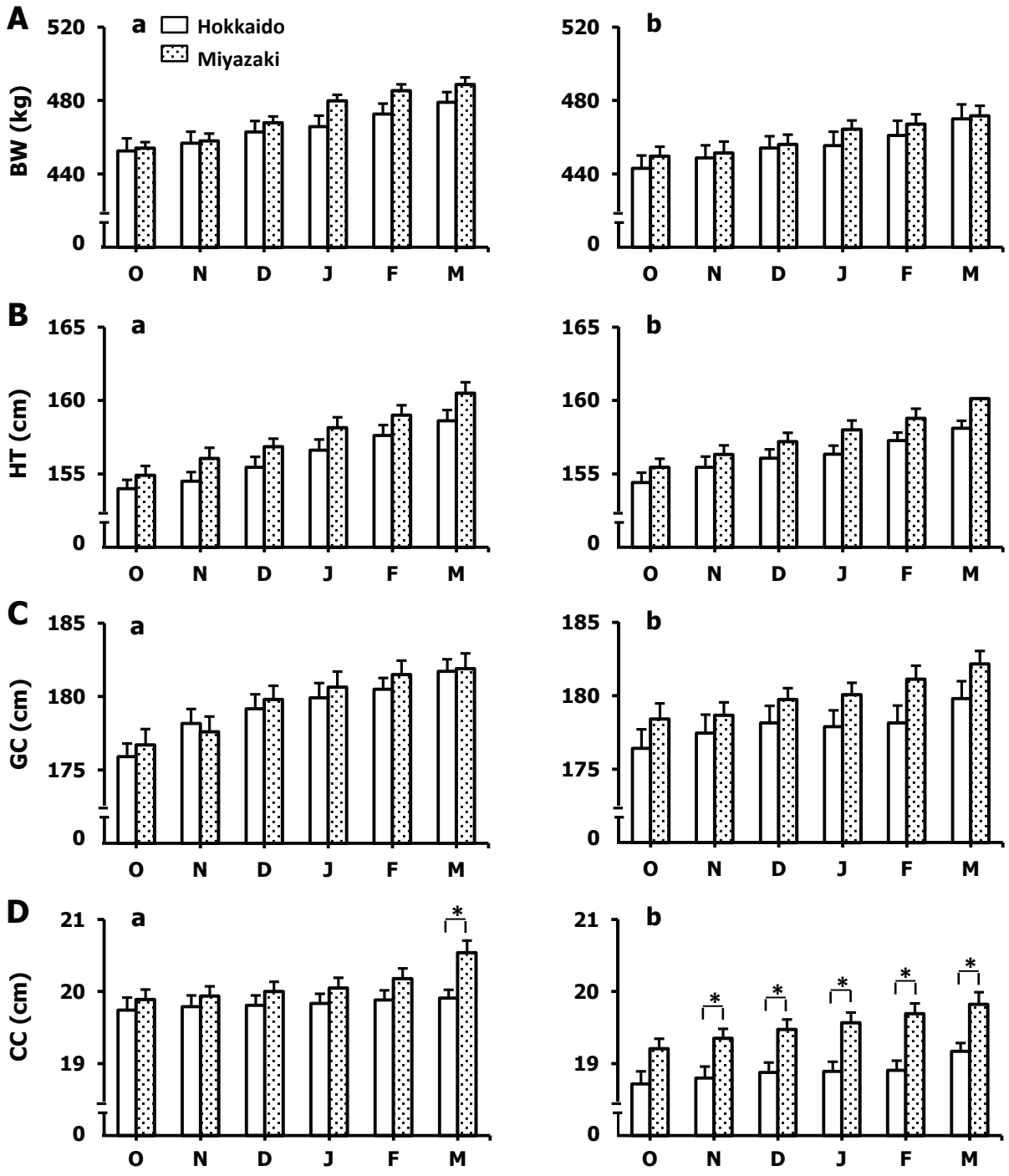


Fig. 4.4 Means of (A) body weight (BW), (B) height (HT), (C) girth (GC) and (D) cannon bone (CC) circumferences compared between Hokkaido (□) and Miyazaki (⚀) in colts (a) and fillies (b) under natural condition from October to March. Each value is expressed as the mean ± SEM. * Denoted the significant differences between different groups in each period and sex (P<0.05).

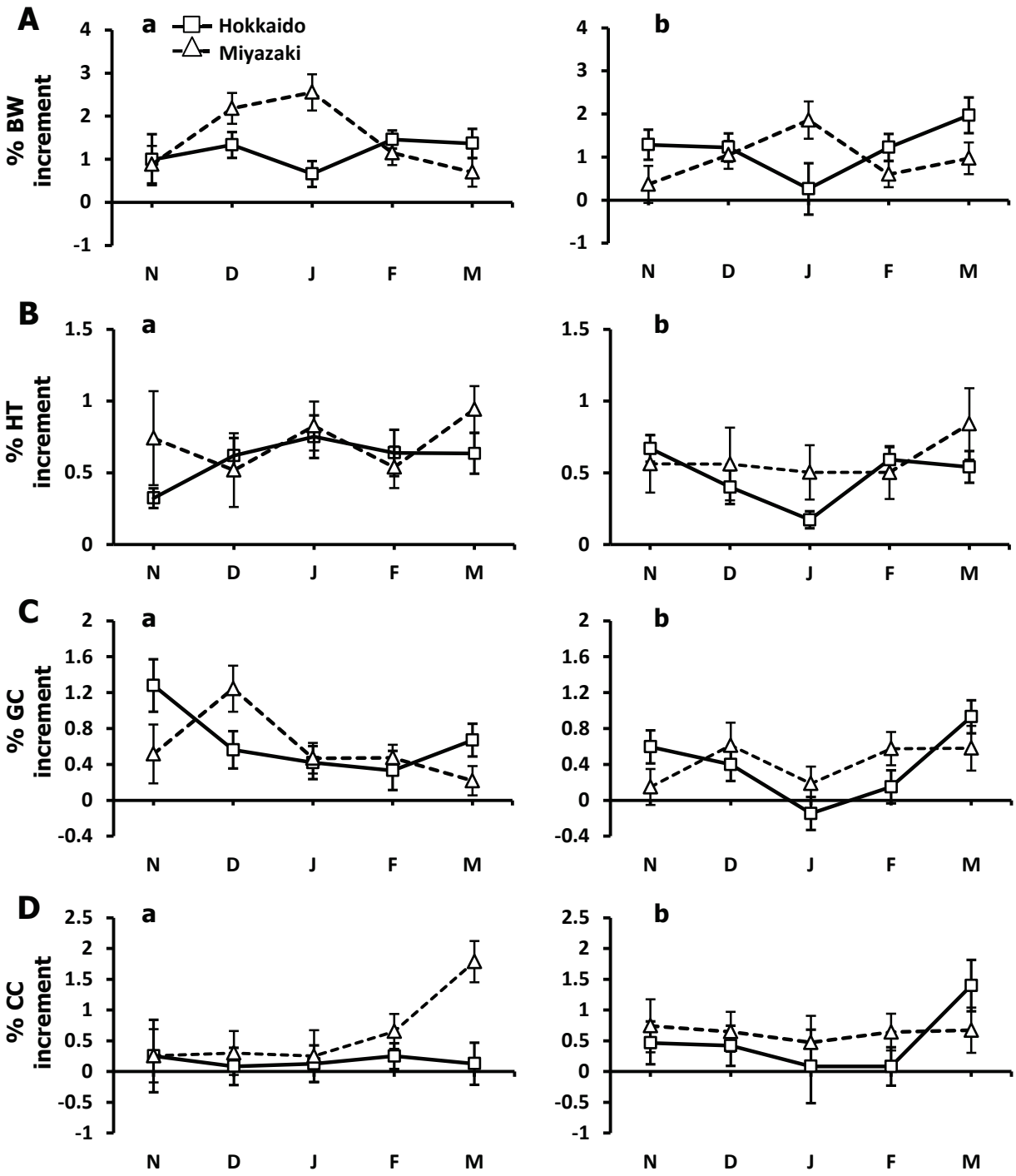


Fig. 4.5 Increment percent (A) body weight (BW), (B) height (HT), (C) girth (GC) and (D) cannon bone (CC) circumferences compared between Hokkaido (-□-) and Miyazaki (--△-) in colts (a) and fillies (b) under natural condition from November to March. Each value is expressed as the mean ± SEM.

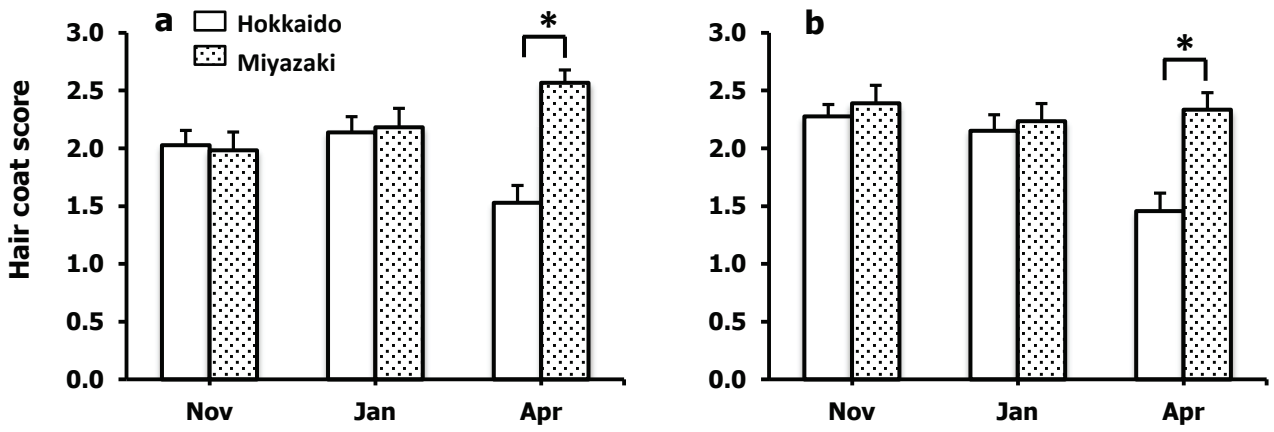


Fig. 4.6 Comparison of hair coat scores between Hokkaido (□) and Miyazaki (⚀) in colts (a) and fillies (b) at November, January and April under natural condition. Each value is expressed as the mean ± SEM. * Denoted the significant differences between groups in each period and sex (P<0.05).

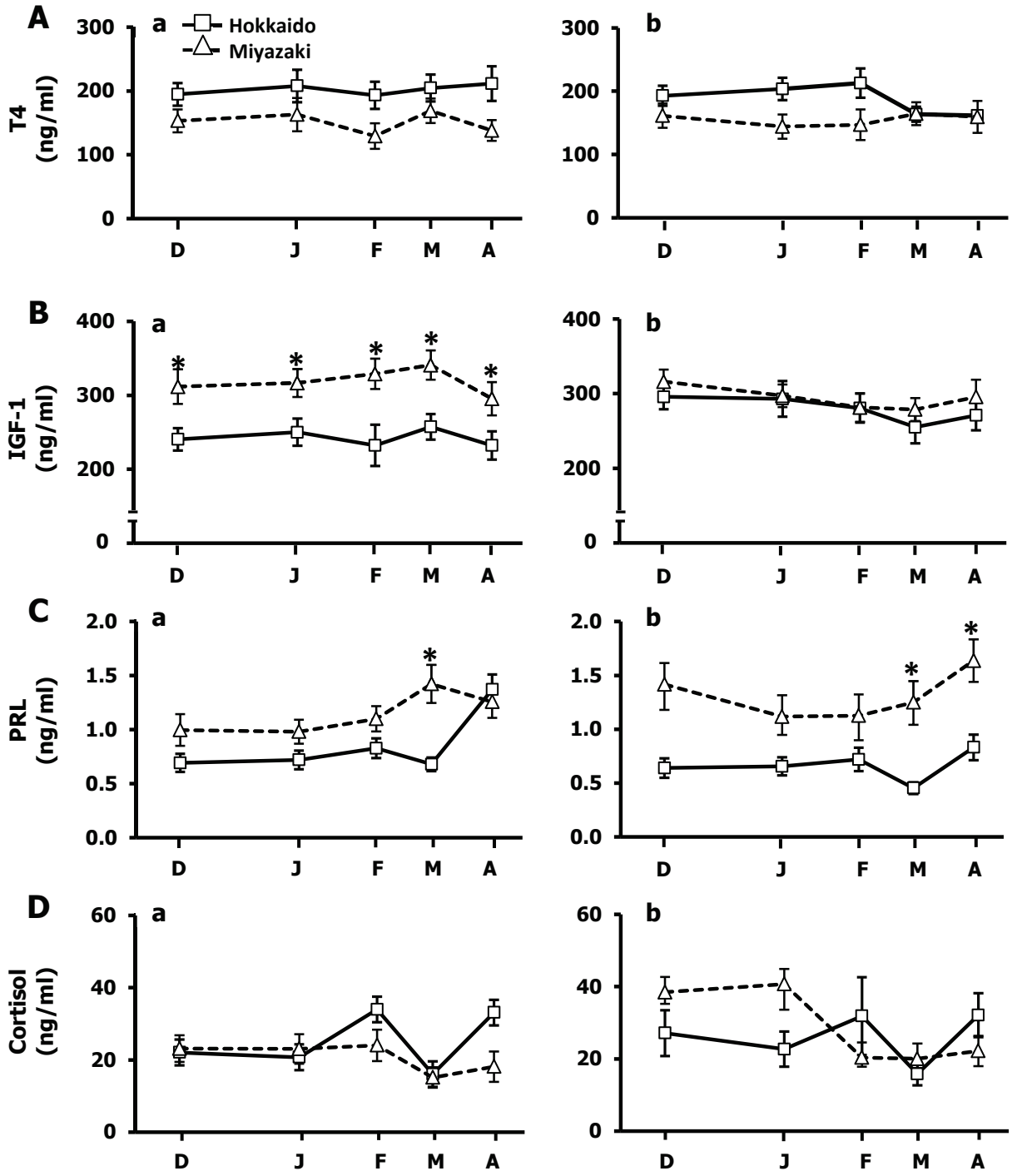


Fig. 4.7 Comparison of circulating T4 (A), IGF-1 (B), PRL (C) and Cortisol (D) concentrations (ng/ml) between Hokkaido (-□-) and Miyazaki (-△-) in both colts (a) and fillies (b) under natural condition from December to April. Values are expressed as mean ± SEM. * Denotes the significant differences at $P<0.05$ between different groups in each period and sex.

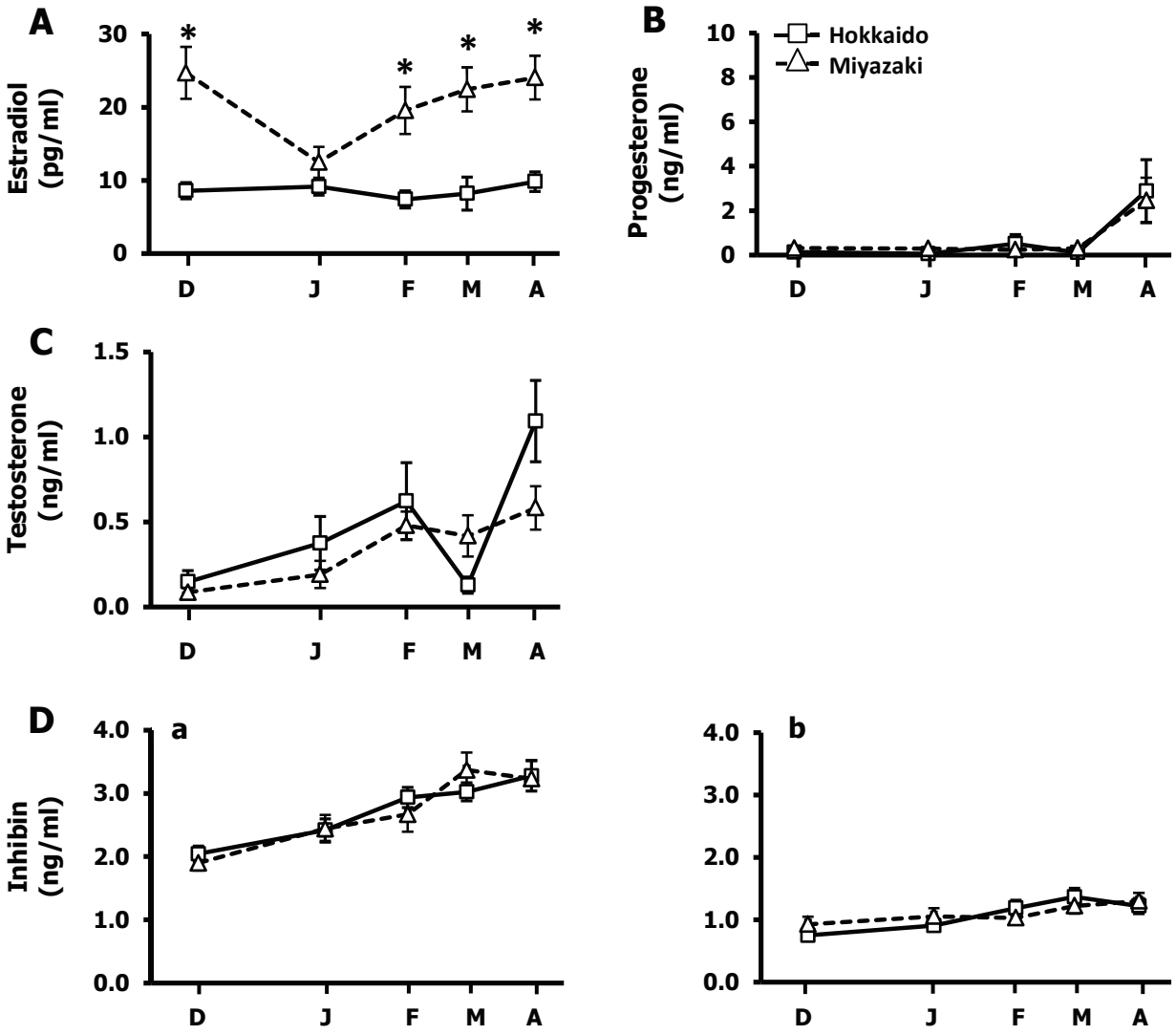


Fig. 4.8 Comparison of circulating estradiol-17β (A) and progesterone (B) in fillies, testosterone (C) in colts and ir-inhibin (D) in both colts (a) and fillies (b) concentrations between Hokkaido (-□-) and Miyazaki (--△-) under natural condition from December to April. Values are expressed as mean ± SEM. * Denotes the significant differences at $P<0.05$ between different groups in each period and sex.

Chapter 5

Effect of light supplementation on metabolic and reproductive endocrine functions and body physical growth in Thoroughbred trained yearling horses in different climate conditions

5.1 Background

Typically, horse is seasonally long-day breeder. Photoperiod influences on onset of puberty and reproductive functions in male and female horses. During spring and summer; long-day period, reproductive activity is the highest while the lowest in shortened day length of autumn and winter. The functions of testes in stallions (Dhakal et al. 2011, 2012; Nagamine et al. 1998) and of ovary in mares (Nagata et al. 1998; Dhakal et al. 2012; Nagy et al. 2000; Nishikawa 1959) are activated by circulating gonadotropins and gonadal hormones via pineal gland and hypothalamic-pituitary-gonadal (HPG) cascade. Artificial light supplementation is widely used to extend daylight hour in clinically healthy and anovulatory broodmares and enhances reproductive functions in stallions during short-day periods or non-breeding season (Nishikawa, 1959). In northern part of Japan, Hokkaido is set on temperate climate zone and well known area for Thoroughbred horse breeding whereas Miyazaki, which is located in the south of Japan, belongs to subtropical climate zone. Furthermore, Hokkaido has day length shorter than in Miyazaki through autumn and winter season. According to the results of comparison in horses which were rasied between north and south under natural condition (see Chapter 4) revealed that Hokkaido yearling horses had growth rate and reproductive development slower than horses in Miyazaki. Long-day extended photoperiod treatment was successful to improve reproductive activities in adult

horses (Sharp and Cleaver 1993; Kenny et al. 1975). Nevertheless, the effect of light supplementation on metabolic and reproductive functions in yearling horse is quite limited and also its mechanism on growth in yearlings is still unclear.

The objectives of this study were to 1) investigate the effect of light supplementation on changes in metabolic and reproductive hormones and body physical growth in Thoroughbred trained yearling horses which were raised in different climate, Hokkaido and Miyazaki of Japan.

5.2 Materials and methods

5.2.1 Animals

During two year seasons (2012-2013 and 2013-2014), total 160 Thoroughbred trained yearling horses, were used from 1 year old of age at September (autumn) to less than 2 year of age at April (spring), in 2 facilities of the JRA, Hidaka Training and Research Center in Hokkaido (temperate north, latitude 42.2° and longitude 142.8°) or Miyazaki Training Yearling Farm in Miyazaki (subtropical south, latitude 31.9° and longitude 131.4°). Horses in each place were randomly divided into 2 groups; control and light supplementation (LS) groups. Twenty-five control (12 colts, 13 fillies), 91 LS (44 colts, 47 fillies) in Hokkaido and 22 control (10 colts, 12 fillies), 22 LS (11 colts, 11 fillies) in Miyazaki were used, respectively. (Diets and exercise program see Chapter 2).

For control groups, all horses were the same as those horses which were used in Chapter 4. The study in this chapter was to compare metabolic and reproductive hormonal and body growth parameters between control and LS groups.

5.2.2 Light supplemental program

In LS groups, artificial light supplementation was conducted by using timer-linked 100 watt white light bulb which was set in the ceiling of the horse stall (3.6 x 3.6 m). The light supplementation was performed 2 times per day as follow;

- At morning, starting before sunrise from 5.30 h to 8.00 h
- At evening, starting before sunset from 16.30 h to 20.00 h

The photoperiod was extended equally as summer in 14.5 h daylight and 9.5 h of dark period (Northern Hemisphere) from December 25th to April 16th in each year for two year seasons. All horses in control groups only exposed to natural light and had no receive the artificial light supplementation throughout the experimental period.

5.2.3 Growth parameters and hair coat conditions

Body weight, body height, girth and cannon bone circumferences were measured and calculated for the increment percent of all parameters as described in Chapter 4.

5.2.4 Endocrine parameters

For endocrine function, circulating total T4, Insulin-like growth factor-I (IGF-1), prolactin (PRL), immunoreactive (ir-) inhibin and cortisol levels were measured by radioimmunoassay. The measurement of circulating sex steroid hormones, estradiol-17β and progesterone in fillies, and testosterone in colts were performed by time-resolved fluoroimmunoassay (DELFIA, Eu-Labelling kit, PerkinElmer, USA) as described in Chapter 4.

5.2.5 Determination of ovarian and testicular function

First expected activity of ovary in fillies and testis in colts were arbitrarily defined as described in Chapter 4.

5.2.6 Statistical analysis

All statistical analysis was performed using R software. The results were defined as mean ± standard errors of the means (SEM). Raw data of all parameters in control groups were the same as in Chapter 4, but data analysis in the present chapter was different from the previous study by comparison between control and LS groups. The differences in means of all parameters between control and LS groups and the repeated-measures data were analyzed using the generalized least squares (GLS) with adjustments by Bonferroni's multiple comparison tests. The significance level was set at alpha = 0.05.

5.3 Results

All of results in this study mainly focused on the differences of body growth, metabolic and reproductive hormonal levels between LS and control horses in each place.

Growth parameters

The monthly means of body weight, body height, girth and cannon bone circumferences comparing between LS and control groups are shown in Fig. 5.1A-D for Hokkaido and Fig. 5.2A-D for Miyazaki. Body weight, body height and girth circumference had no significant differences between LS and control groups in both Hokkaido and Miyazaki. However, body weight, height and girth in LS Hokkaido colts and fillies tended to be higher than the controls (Fig 5.1A-C). In Miyazaki, even though colts and fillies in LS groups had body weight, height and girth lower than the controls at the starting of experiment, those three parameters increased nearly to the levels of the controls eventually, especially in fillies (Fig. 5.2A-C). Cannon bone circumferences in both LS Hokkaido and Miyazaki colts were not different from controls significantly (Fig. 5.1D; a and 5.2D; a). In fillies, cannon bone

circumferences in LS Hokkaido groups were significantly higher than the controls (Fig. 5.1D; b) while Miyazaki showed higher trend without significant differences (Fig. 5.2D; b).

For increment percent of 4 growth parameters, both Hokkaido and Miyazaki, the increment percent of body weight (Fig. 5.3A and 5.4A), height (Fig. 5.3B and 5.4B), girth (Fig. 5.3C and 5.4C) and cannon bone (Fig. 5.3D and 5.4D) circumferences were not clearly different between LS and control groups in both colts and fillies. Nevertheless, the increment percent of body weight and girth in LS groups of both Hokkaido and Miyazaki tended to be higher than the controls during February and/or March (Fig. 5.3A, C and 5.4A, C).

Furthermore, means of increment percent accounted from November to March for 2 year seasons had no significant differences between LS and control groups of Hokkaido (Fig 5.5A) and Miyazaki (Fig 5.5B) in any growth parameters. Nevertheless the increments of body weight and cannon circumferences in Hokkaido colts, girth in Hokkaido fillies, and body weight and girth in Miyazaki fillies seemed to be higher in LS groups than the controls.

Hair coat conditions

The hair coat condition scores comparing between LS and control groups are shown in Fig. 5.6. There were no significant differences in hair coat scores between LS and control groups in November and January on both Hokkaido and Miyazaki colts and fillies. In April, the hair coat scores in LS Hokkaido colts and fillies were increased and significantly higher than the controls (Fig. 5.6A), whereas Miyazaki horses did not show any significant differences between LS and control groups (Fig. 5.6B)

Endocrine functions

Comparisons of circulating total T4, IGF-1, PRL and cortisol between LS and control groups are demonstrated in Fig. 5.7 for Hokkaido and Fig. 5.8 for Miyazaki. Circulating T4

concentrations had no significant differences between LS and control groups in Hokkaido colts (Fig. 5.7A; a) and Miyazaki colts and fillies (Fig. 5.8A; a-b). In Hokkaido fillies, the T4 concentrations were significantly higher in LS group than the control in early April (Fig. 5.7A; b). For IGF-1, only LS Hokkaido colts showed the levels significantly higher than control throughout the periods (Fig. 5.7B; a). No significant differences of IGF-1 levels between control and LS groups were noted in Hokkaido fillies (Fig. 5.7B; b) and Miyazaki colts and fillies (Fig. 5.8B; a-b). Circulating prolactin, LS groups of Hokkaido (Fig. 5.7C; a-b) and Miyazaki (Fig. 5.8C; a) had PRL concentrations higher than the controls significantly. In Hokkaido, the prolactin level rose in early April in control groups, but it increased in late January in LS groups (Fig. 5.7C). For Miyazaki, PRL concentration rose around mid-March in control groups, whereas it increased at late January in LS groups (Fig. 5.8C).

For reproductive endocrine changes, circulating estradiol-17β concentrations increased in LS Hokkaido fillies from late January and continued constantly to April, but were not significantly different to the controls (Fig. 5.9A). In Miyazaki, no significant differences of estradiol-17β concentration were found between LS and control groups (Fig. 5.10A). Progesterone levels in blood circulation in fillies were significantly higher in LS groups than the controls in both Hokkaido (Fig. 5.9B) and Miyazaki (Fig. 5.10B). Moreover, LS Hokkaido and Miyazaki fillies seemed to have first ovarian activity earlier (late February) than the controls (early April) at the progesterone level over 1 ng/ml. For testosterone, the LS groups of Hokkaido and Miyazaki colts tended to show less elevated levels but there were no significant differences when compared to the controls (Fig. 5.9C and 5.10C). First testicular activities in both Hokkaido and Miyazaki groups were earlier than those of controls at testosterone level reached or higher 0.5 ng/ml. No significant differences of ir-inhibin levels between LS and control groups in both colts and fillies of Hokkaido and Miyazaki (Fig. 5.9D

and 5.10D). In addition, there were no significant differences in circulating cortisol concentrations between LS and control groups in Hokkaido fillies (Fig. 5.7D; b) and Miyazaki colts and fillies (Fig. 5.8D, a-b). However, the cortisol levels in LS Hokkaido colts were lower significantly than the controls in late February (Fig. 5.7D; a).

5.4 Discussion

Under light supplementation, the present study found that the monthly means and increment percent in almost growth parameters tended to be higher in LS groups of both Hokkaido and Miyazaki compared to the controls. Also, as to increment percent in growth parameters during 5 months of experimental period, the increments of body weight and cannon circumference in Hokkaido colts, girth in Hokkaido fillies, and body weight and girth in Miyazaki fillies tended to be higher in LS groups than the controls which consistent to previous study (Suzuki et al. 2015). From these results, light supplementation seemed to increase the body growth in some parameters. Generally, growth was influenced by several factors, nutrition, heredity, exercise and hormone mainly involved to body development (Reed et al. 2004). These results suggested that artificial lighting was able to support or enhance growth in young horses.

Regarding hair coat condition, there were no significant differences in hair coat scores between LS and control horses in November and January in both Hokkaido and Miyazaki. Finally, the present study clearly demonstrated that LS groups in Hokkaido both colts and fillies had hair coat scores in April better than control groups, whereas no significant differences were observed in Miyazaki. This suggested that LS supplementation helped to improve hair coat condition in Hokkaido horses obviously. Consistently, previous reports described that extended photoperiod treatment stimulated molting of winter hair

coat (Nishikawa 1959; Kooistra and Ginther 1975; Nambo et al. 2010). The results of hair coat condition consistent to PRL levels found that LS Hokkaido and Miyazaki in both colts and fillies had PRL levels higher than the controls. The results of present study were consistent with previous publications reporting that administration of prolactin (Thompson et al. 1997) or dopamine antagonist (Thompson et al. 1997; Donadeu and Thompson 2002) induced winter coat molting in horses. These suggested that light supplementation promoted earlier increasing of PRL concentration which might be involved in hair shedding in yearling horses.

For endocrine changes, the present study showed that no significant difference of T4 levels between LS and control groups in Hokkaido colts and Miyazaki colts and fillies. However, the higher trend of T4 concentrations were noted in LS Hokkaido fillies from March to April since T4 decreased in control fillies. In other species photoperiod affected to thyroid activity, for example peak thyroid hormone concentrations in plasma of goats were found in the increasing daylength period of spring while the lowest levels were recorded during decreasing daylight of early autumn (Rhind et al. 2000; Souza et al. 2002; Todini et a;. 2006; Todini et al. 1992). These present results cannot definitely rule out the possibility of supplemental lighting-induced change in blood thyroid hormone in horses because hormone concentrations in blood circulation are only representative of the net balance between rates of synthesis and metabolism. For IGF-1, this study did not show any differences in circulating IGF-1 concentrations between LS and controls groups in Hokkaido fillies and Miyazaki in both genders. Only LS Hokkaido colts had significant higher IGF-1 levels than the controls. However the artificial light-induced increasing of IGF-1 was not presumed because the beginning IGF-1 levels were higher than the controls before light supplementation and patterns of fluctuation were similar to those of controls. These results suggested that IGF-1

concentrations in blood circulation might obtain from whole body and was probably not sensitive response in yearling horses. Consistent to the previous study reported that localization of IGF-1 and its receptor were stronger in post-pubertal than pre-pubertal male horses (Yoon and Roser 2010).

The present study showed that light supplementation helped to raise circulating PRL concentrations and also advanced the first ovarian and testicular activity in fillies and colts of both Hokkaido and Miyazaki, respectively. PRL is secreted to blood circulation with greater level during longer photoperiod and lower level in shortened daylight. It is mainly associated with mammary growth, lactation, and also responsible for hair shedding. Recently, PRL plays diverse role in reproduction in several species including male and female horses (Brinsko et al. 2011). Previous studies reported that PRL and its receptor were found in equine follicular fluid, ovarian follicles and corpus luteum (CL) (Daoud and Ezzo 2014; King et al. 2010; King et al. 2014; Oberhaus et al. 2015). In male horses, circulating PRL concentration increased in long-day season as the female (Curlewis 1992). Previous research suggested that PRL involved in testosterone secretion via the stimulation of LH receptor expression in rodent Leydig cells (Hair et al. 2002). Our results in accordance with those previous studies suggested that the light-induced PRL increase might hasten gonadal function in yearlings by the increase in number of LH receptor in granulosa/theca, CL of ovary and Leydig cells of testis (Jones and Hsueh 1981; Van Straalen and Zeilmaker 1982). According to growth results in the present study, PRL was probably involved in development of the body indirectly supported by previous studies noted that calcium absorption in intestine was promoted by PRL (Charoenphandhu et al. 2010; Suntornsaratoon et al. 2010) and that expression of PRL receptor was found in epiphyseal plate (Suntornsaratoon et al. 2010).

Regarding the reproduction, estradiol-17β and testosterone concentrations tended to elevate in LS Hokkaido fillies, and colts in both Hokkaido and Miyazaki, respectively compared to the controls. Also, first ovarian and testicular activities designated by the levels of progesterone and testosterone were obviously earlier in LS fillies and colts of both Hokkaido and Miyazaki, respectively in accordance with previous studies describing that extended photoperiod advanced ovulation in young female horses (Suzuki et al. 2015; Kunii et al. 2015). These suggested that artificial light supplementation hastened gonadal functions in yearling horses by stimulation of PRL secretion and hypothalamic-pituitary-gonadal axis.

For cortisol, our study showed no clear differences in cortisol concentrations between LS and control groups in both Hokkaido and Miyazaki. These suggested that light supplementation did not have negative effect such as stress on horses. However, in February the cortisol levels in LS Hokkaido colts and fillies tended to be lower than the controls which were high from exercising program (see in Chapter 4).

All of results regarding the effect of light supplementation on the body growth, hair coat condition and endocrine functions clarified that the light-induced prolactin increasing via stimulation of pineal gland and hypothalamic-pituitary-gonadal axis, leading to hasten gonadal function, advance winter coat shedding, and improve to growth and development in yearling horses.

In conclusion, light supplementation was able to improve growth and reproductive function in both colts and fillies in Hokkaido and Miyazaki.

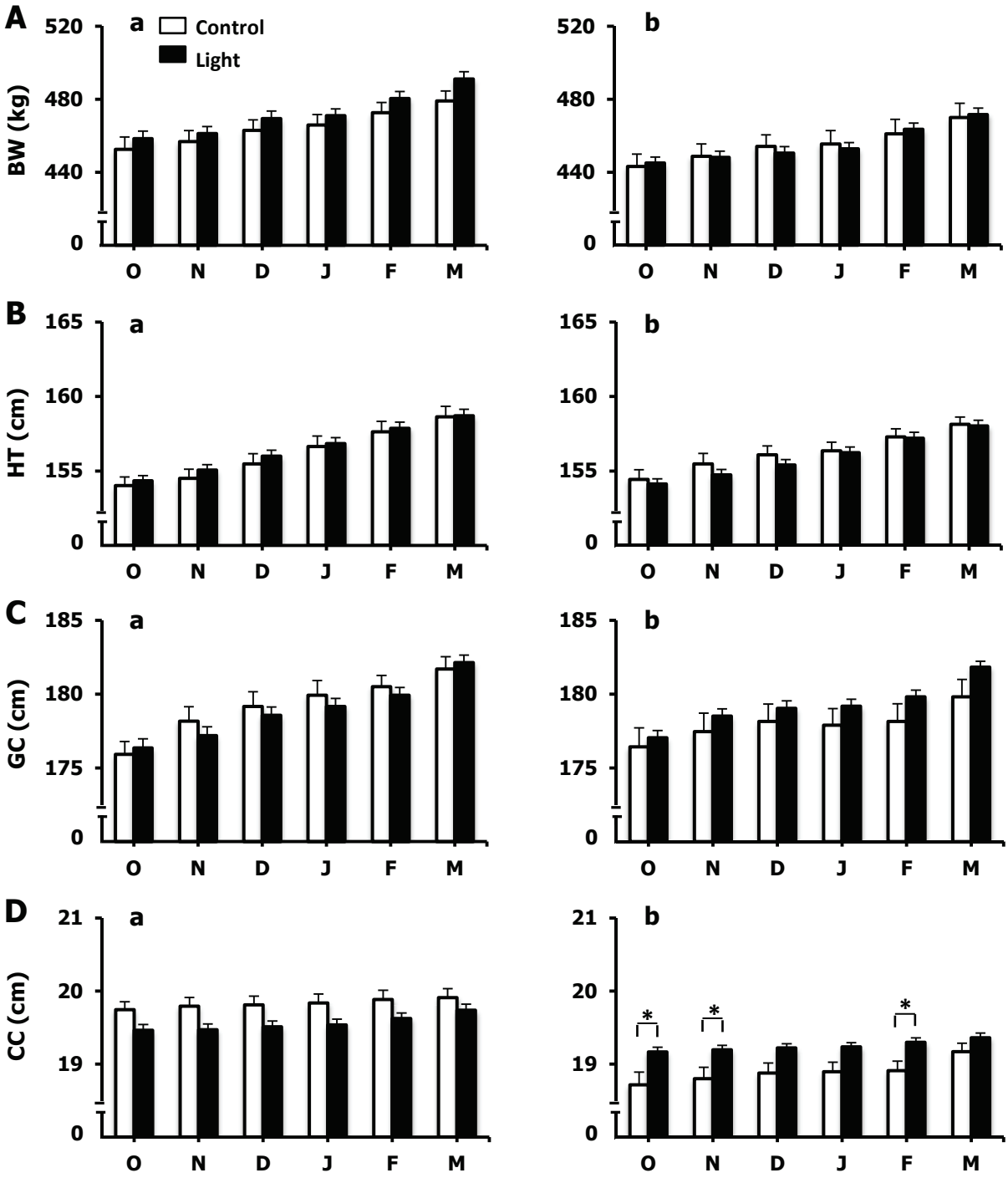


Fig. 5.1 Mean of (A) body weight (BW), (B) height (HT), (C) girth (GC) and (D) cannon bone (CC) circumferences compared between control (□) and LS (■) groups in Hokkaido colts (a) and fillies (b) from October to March. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in the same period in each sex (P<0.05).

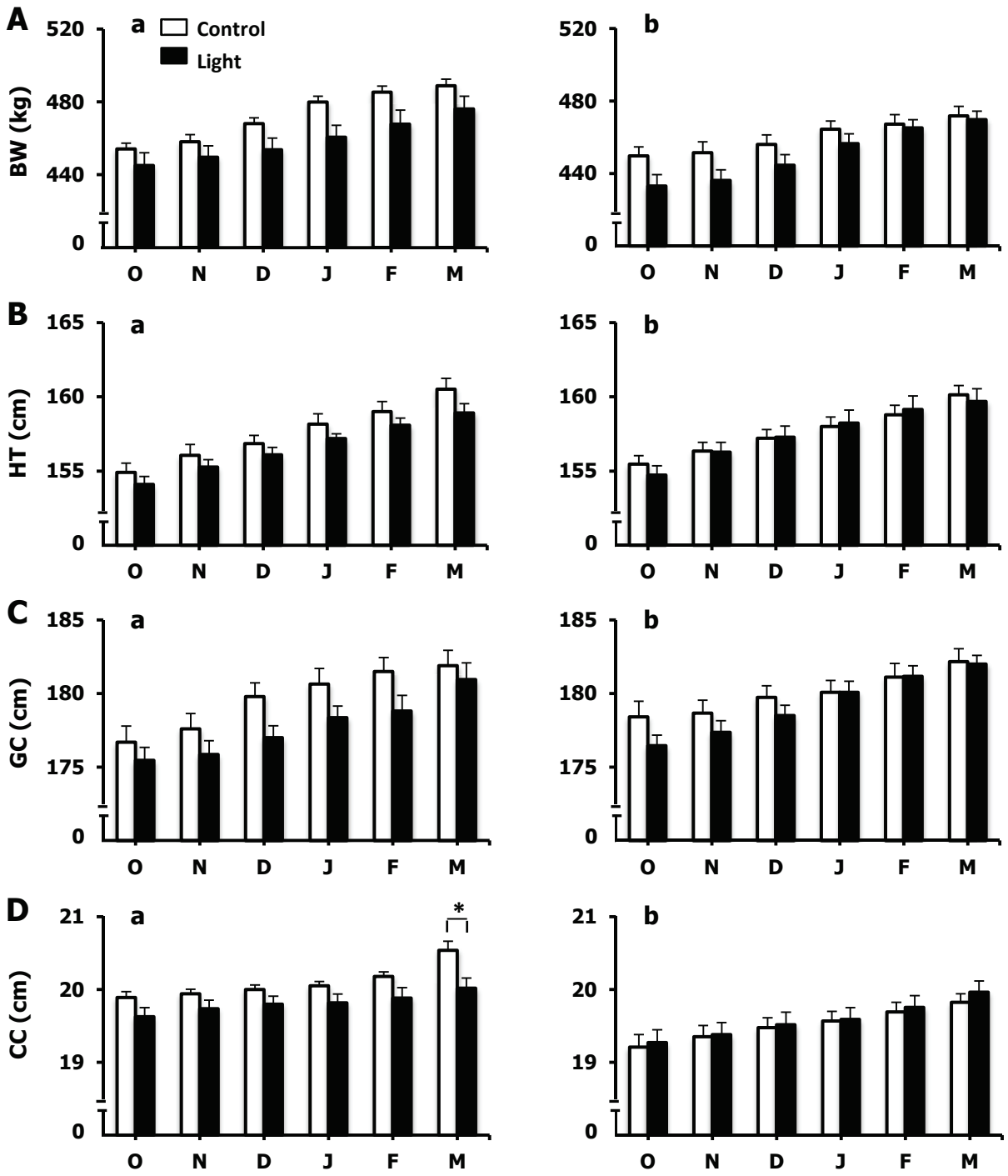


Fig. 5.2 Mean of (A) body weight (BW), (B) height (HT), (C) girth (GC) and (D) cannon bone (CC) circumferences compared between control (□) and LS (■) groups in Miyazaki colts (a) and fillies (b) from October to March. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in the same period in each sex (P<0.05).

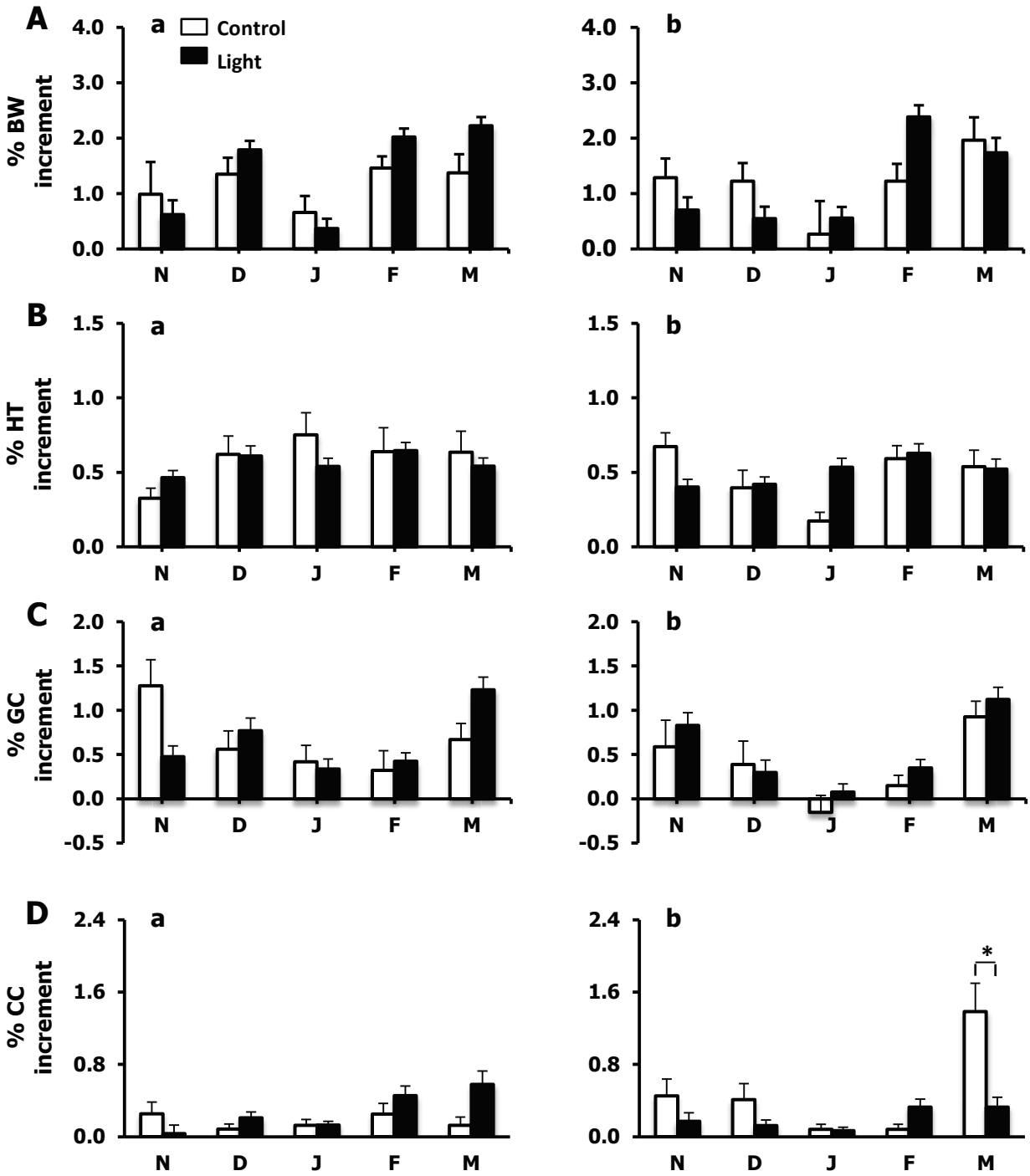


Fig. 5.3 Increment percent of (A) body weight (BW), (B) height (HT), (C) girth (GC) and (D) cannon bone (CC) circumferences compared between control (□) and LS (■) groups in Hokkaido colts (a) and fillies (b) from November to March. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in the same period in each sex (P<0.05).

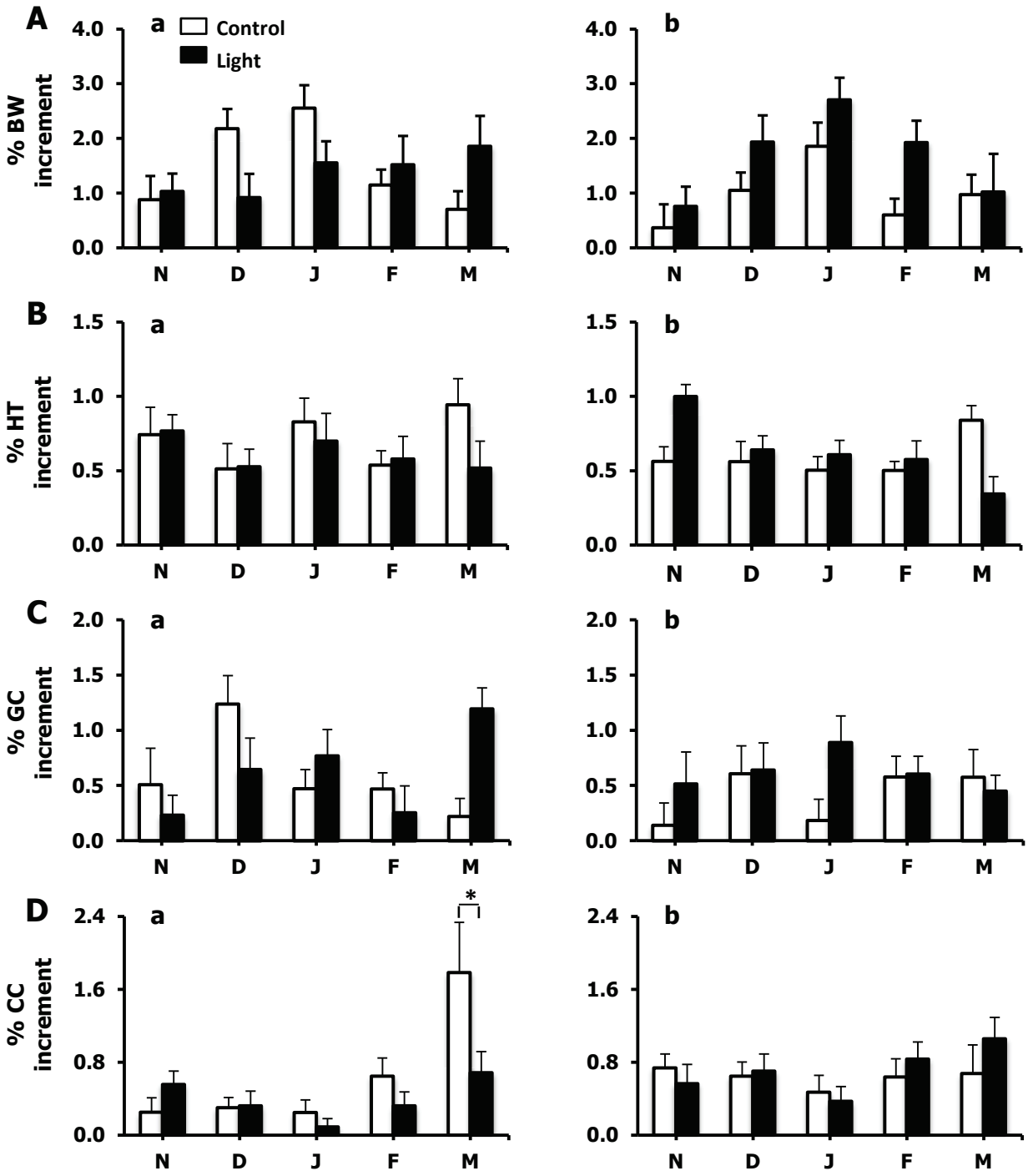


Fig. 5.4 Increment percent of (A) body weight (BW), (B) height (HT), (C) girth (GC) and (D) cannon bone (CC) circumferences compared between control (□) and LS (■) groups in Miyazaki colts (a) and fillies (b) from November to March. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in the same period in each sex (P<0.05).

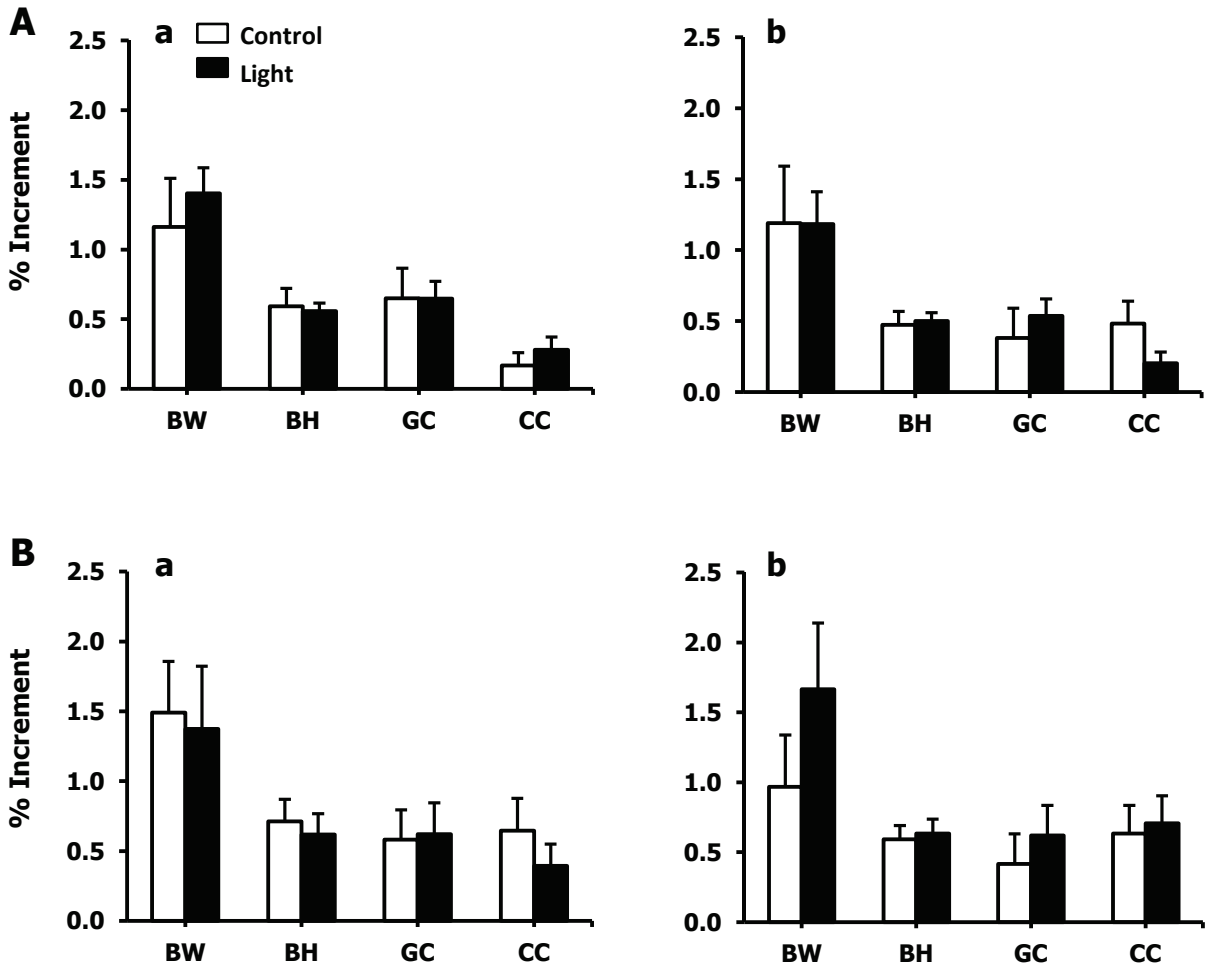


Fig. 5.5 Increment percent of growth parameters during 5 months of experimental periods. Body weight (BW), body height (BH), girth (GC) and cannon bone (CC) circumferences were compared between control (□) and LS groups (■) in both colts (a) and fillies (b) of Hokkaido (A) and Miyazaki (B). Values are expressed as mean ± SEM.

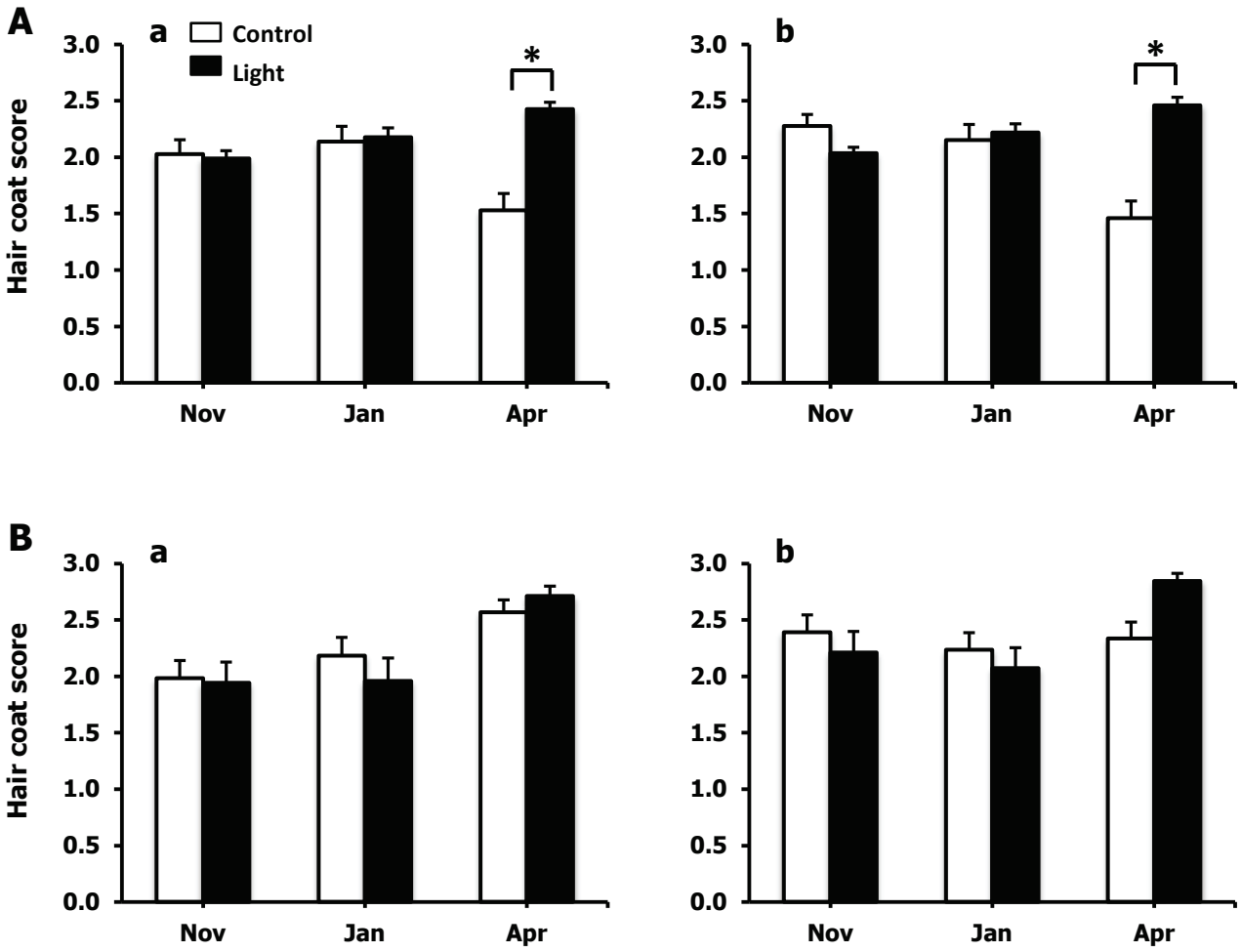


Fig. 5.6 Scores of hair coat condition comparing between control (□) and LS groups (■) in both colts (a) and fillies (b) of Hokkaido (A) and Miyazaki (B). Values are expressed as mean ± SEM. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in the same period in each sex (P<0.05).

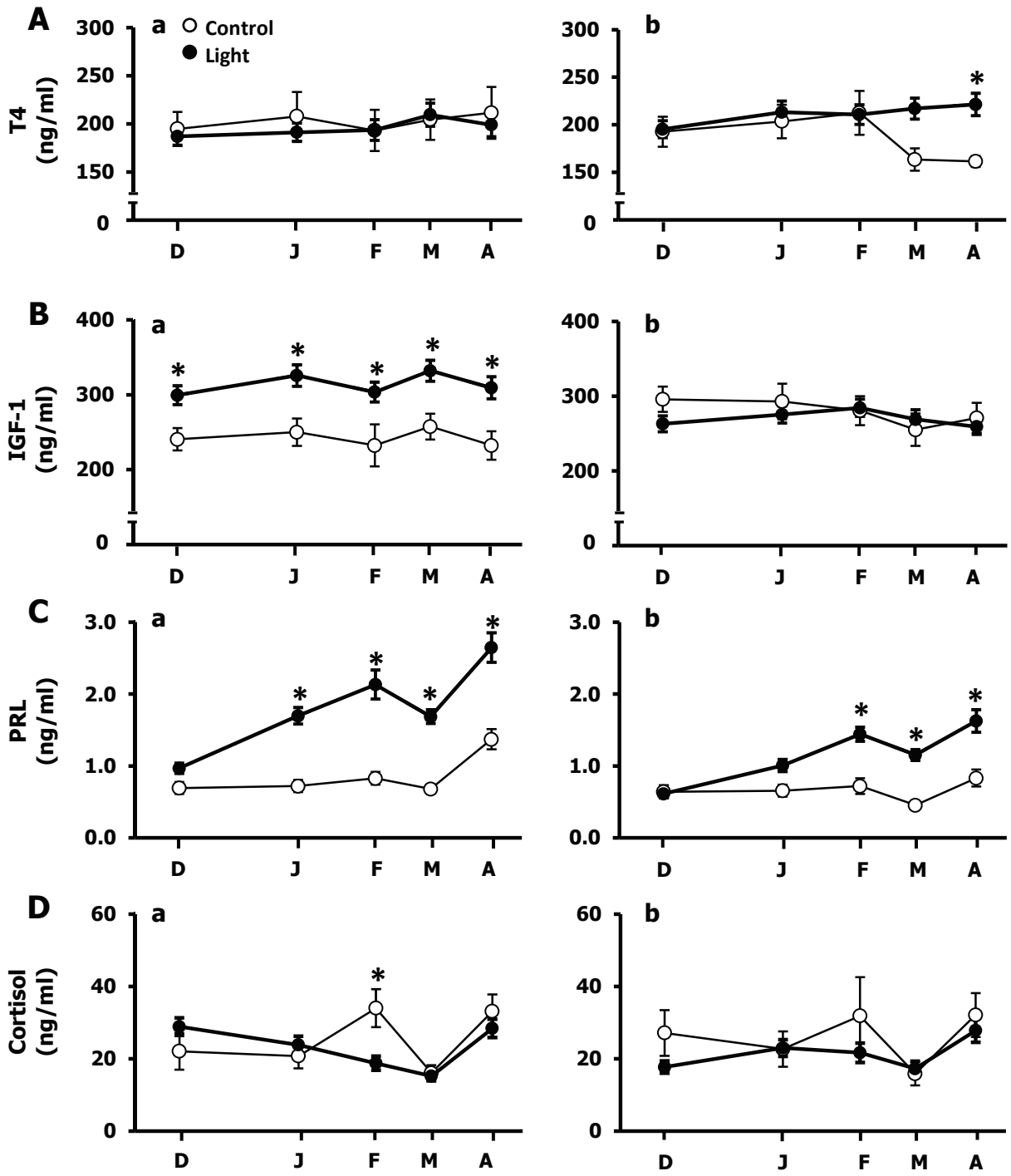


Fig. 5.7 Circulating total T4 (A), IGF-1 (B), PRL (C) and Cortisol (D) concentrations in both colts (a) and fillies (b) of Hokkaido comparing between control (○) and LS treatment groups (●) from December to April. Values are expressed as mean ± SEM. * Denotes the significant differences between different treatment groups in the same period in each sex (P<0.05).

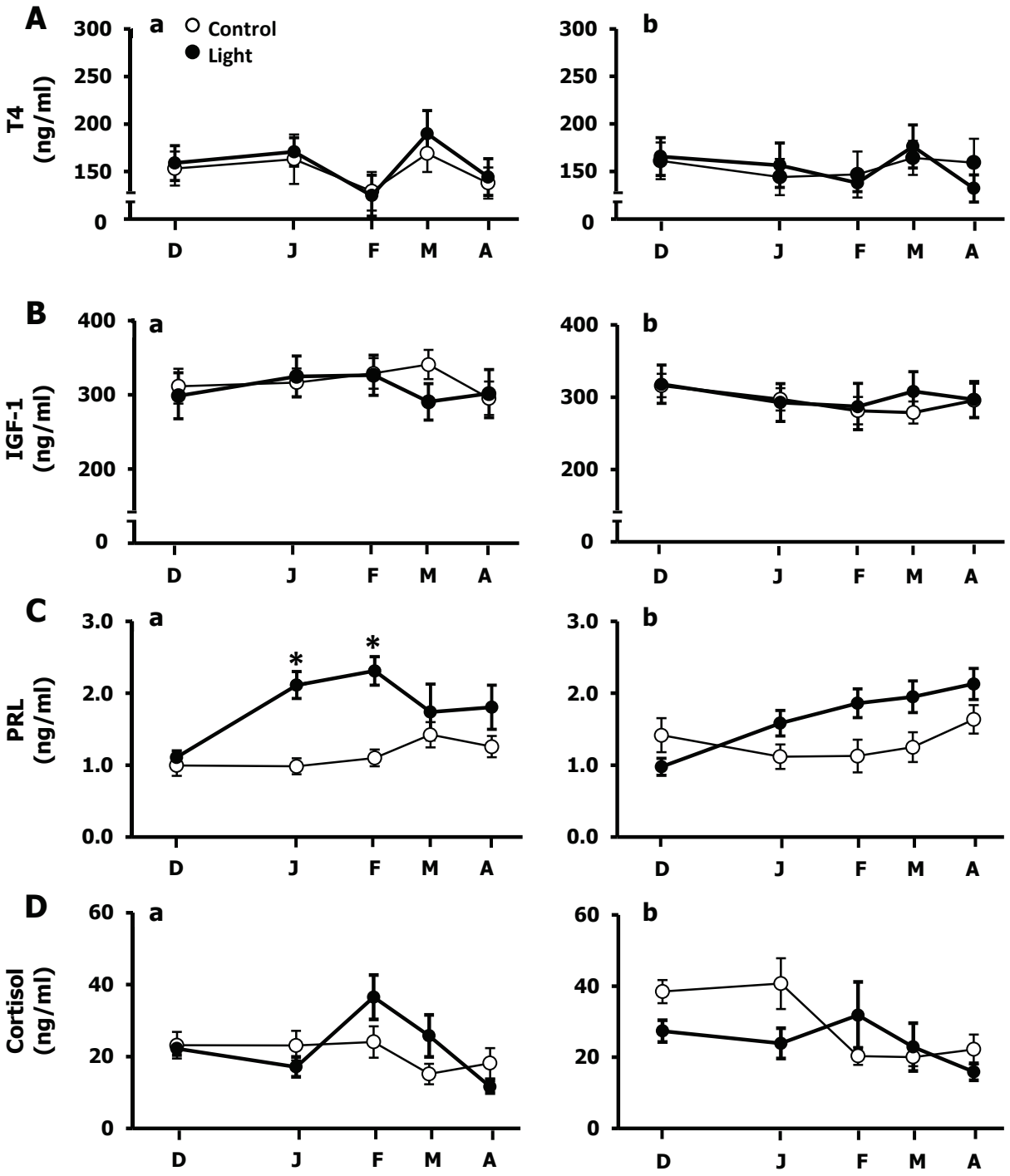


Fig. 5.8 Circulating total T4 (A), IGF-1 (B), PRL (C) and Cortisol (D) concentrations in both colts (a) and fillies (b) of Miyazaki comparing between control (○) and LS groups (●) from December to April. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in the same period in each sex (P<0.05).

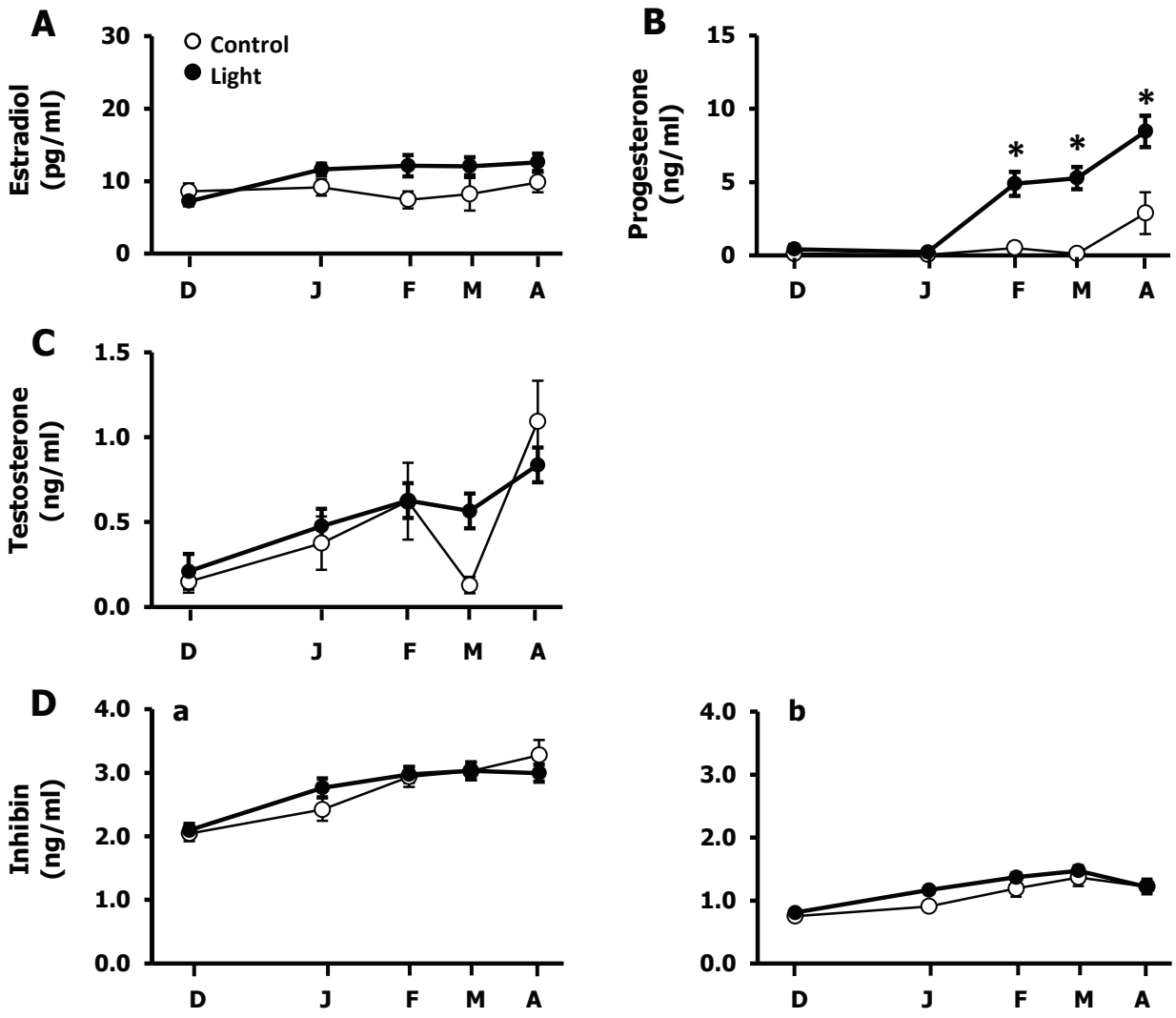


Fig. 5.9 Circulating estradiol-17β (A) and progesterone (B) in fillies, testosterone (C) in colts, and ir-inhibin (D) in both colts (a) and fillies (b) of Hokkaido comparing between control (○) and LS groups (●) from December to April. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in the same period in each sex (P<0.05).

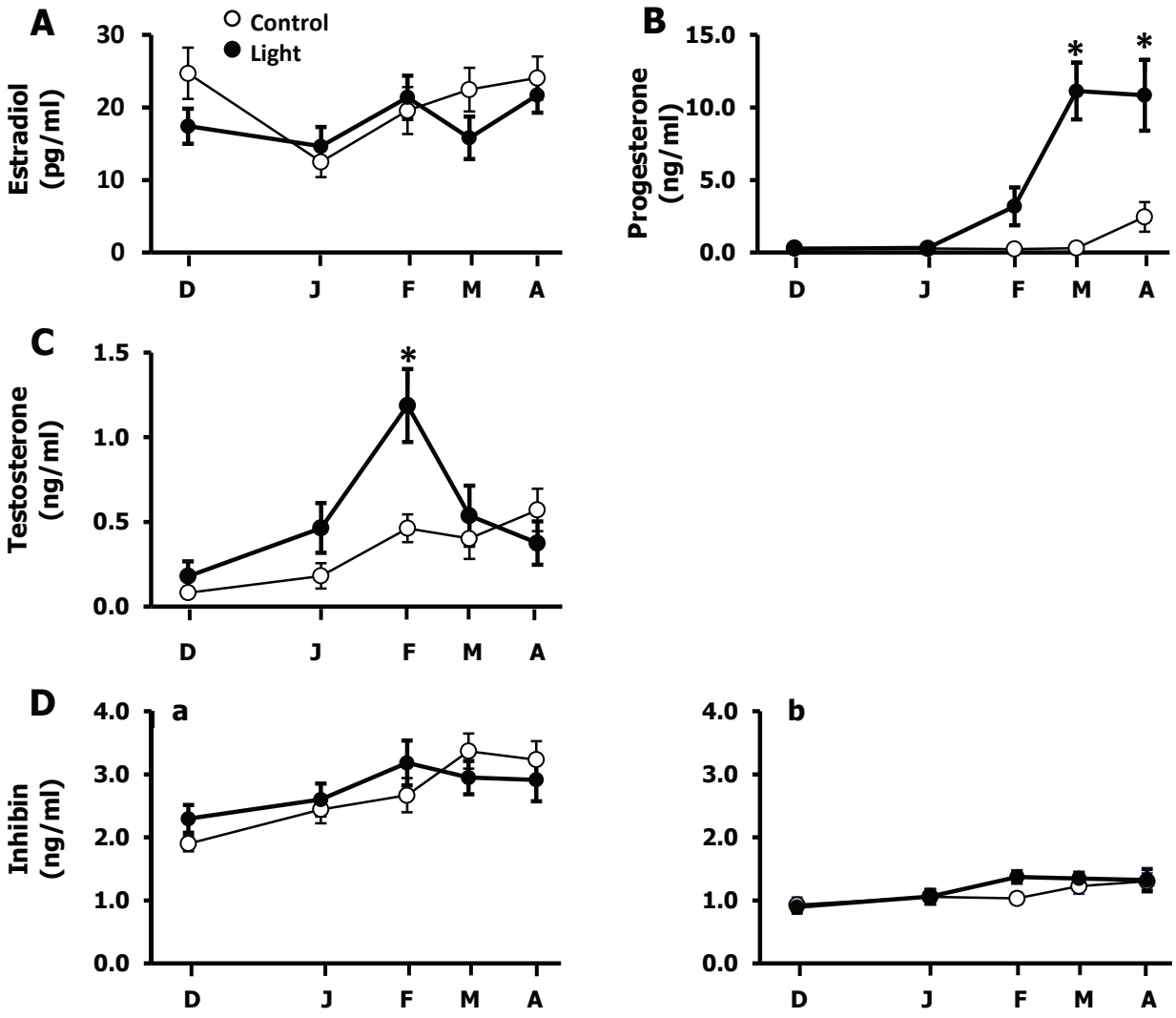


Fig. 5.10 Circulating estradiol-17β (A) and progesterone (B) in fillies, testosterone (C) in colts, and ir-inhibin (D) in both colts (a) and fillies (b) of Miyazaki comparing between control (○) and LS groups (●) from December to April. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in the same period in each sex (P<0.05).

Chapter 6

Comparison of metabolic and reproductive endocrine functions and body physical growth between north and south climates of Japan in Thoroughbred trained yearling horses under light supplementation

6.1 Background

Horses are used by human for many purposes such as agriculture, transportation, athletics, etc. Thoroughbred is a popular horse breed which wildly used for horse racing. In race horse industry, not only fertile reproductive properties are expected for breeder but also required good body physical conformation in order to succeed in racing career. Typically, horses are long-day seasonal breeders which have active ovarian and testicular functions in spring and summer while become inactive during autumn and winter. The previous study reported that some adult horses had prolonged anestrus or subfertility during non-breeding season (Reed et al. 2004). Artificial lighting program has been widely used to enhance reproductive activities in stallion and mare. In Japan, the difference between temperate climate in the north and subtropical climate in the south consistent with our results (see chapter 4) demonstrated that young horses raised in Hokkaido (north) had inferiority to horses reared in Miyazaki (south). According to previous results, artificial lighting was able to advance gonadal functions and probably promote body growth in young horses associated with hormonal regulations (see chapter 5).

Regarding our results in those chapters, therefore this study aimed to compare metabolic and reproductive hormones and body physical growth between Hokkaido and Miyazaki horses under light supplementation condition.

6.2 Materials and methods

6.2.1 Animals, Diets, and Exercise program

Total 113 Thoroughbred trained yearling horses in two year seasons (2012-2013 and 2013-2014) which raised in 2 facilities of the JRA, Hidaka Training and Research Center in Hokkaido (temperate north, latitude 42.2° and longitude 142.8°) or Miyazaki Training Yearling Farm in Miyazaki (subtropical south, latitude 31.9° and longitude 131.4°) were chosen to be subjects in this study. All horses were from 1 year old of age at September in autumn to less than 2 year of age at April in spring season. Ninety-one Hokkaido (44 colts, 47 fillies) and 22 Miyazaki (11 colts, 11 fillies) horses exposed to artificial light supplementation (LS) for 14.5 h daylight and 9.5 h of dark period equally as summer from December 25th to April 16th in each year by using timer-linked 100 watt white light bulb setting in the ceiling of the horse stall.

All LS horses which were used in this study were the same as those horses in Chapter 5. The study in this chapter was to compare metabolic and reproductive hormonal and body growth parameters between Hokkaido and Miyazaki horses under LS condition.

6.2.2 Growth parameters and hair coat conditions

Body weight, body height, girth and cannon bone circumferences were measured and calculated for the increment percent of all parameters as described in Chapter 4.

6.2.3 Endocrine parameters

For endocrine function, circulating total T4, Insulin-like growth factor-I (IGF-1), prolactin (PRL), immunoreactive (ir-) inhibin and cortisol levels were measured by radioimmunoassay. The measurement of circulating sex steroid hormones, estradiol-17β and progesterone in fillies, and testosterone in colts were performed by time-resolved fluoroimmunoassay (DELFIA, Eu-Labelling kit, PerkinElmer, USA) as described in Chapter 4.

6.2.4 Determination of ovarian and testicular function

First expected activity of ovary in fillies and testis in colts were arbitrarily defined as described in Chapter 4.

6.2.5 Statistical analysis

All statistical analysis was performed using R software. The results were defined as mean ± standard errors of the means (SEM). Raw data of all parameters in both control and LS groups were the same as in Chapter 4 and 5, but data analysis in the present chapter was different from the previous studies by comparison between Hokkaido and Miyazaki under LS condition. The differences in means of all parameters between LS Hokkaido and Miyazaki groups and the repeated-measures data were analyzed using the generalized least squares (GLS) with adjustments by Bonferroni's multiple comparison tests. The significance level was set at alpha = 0.05.

6.3 Results

All of results in this study mainly focused on the comparison of body growth, metabolic and reproductive hormonal levels between Hokkaido and Miyazaki horses under LS condition.

Growth parameters

The monthly means of body weight, body height, girth and cannon bone circumferences comparing between Hokkaido and Miyazaki in LS groups are shown in Fig. 6.1. Body weight, height, girth in both colts and fillies (Fig. 6.1A-C), and cannon bone circumference in colts (Fig. 6.1D; a) were not significantly different between LS Hokkaido and Miyazaki groups. This study found that body weight and girth in LS Miyazaki colts and fillies tended to be lower than the LS Hokkaido. For cannon bone circumferences, LS Miyazaki fillies were higher than the Hokkaido significantly in February and March (Fig. 6.1D; b).

The increment percentages of body weight, body height, girth and cannon bone circumferences comparing between Hokkaido and Miyazaki in LS groups are shown in Fig. 6.2. In January, the result showed that body weight and girth increment of LS Hokkaido colts and fillies decreased dramatically and became lower than the Miyazaki. Eventually, the increments increased similarly to Miyazaki levels without significant differences during February to March (Fig. 6.2A, C). For body height (Fig. 6.2B; a-b) and cannon bone circumference (Fig. 6.2D; a) increments, LS Hokkaido horses also showed decreasing trends during January and then the increments increased reaching similar levels of Miyazaki, whereas cannon bone circumference of Miyazaki fillies (Fig. 6.2D; b) were higher in some periods.

Hair coat conditions

The hair coat condition scores comparing between Hokkaido and Miyazaki in LS horses are shown in Fig. 6.3. There were no significant differences of hair coat scores between LS Hokkaido and Miyazaki in both colts and fillies since November to April.

Endocrine functions

Circulating total T4, IGF-1, PRL, cortisol (Fig. 6.4A-D), estradiol-17β, progesterone, testosterone, and ir-inhibin (Fig. 6.5A-D) were compared between LS Hokkaido and Miyazaki horses. In LS, total T4 concentrations in Hokkaido colts and fillies tended to be higher than the Miyazaki throughout the periods with the significant differences in end of February (colts and fillies) and early April (fillies) (Fig. 6.4A; a-b). For the IGF-1 (Fig. 6.4B) and ir-inhibin (Fig. 6.5D) concentrations, there were no significant differences in the levels between LS Hokkaido and Miyazaki in both colts and fillies. Circulating PRL levels in LS Hokkaido colts increased and became higher than the Miyazaki significantly in April (Fig. 6.4C; a), while the increased PRL levels in fillies tended to be lower (Fig. 6.4C; b). Also, LS Hokkaido fillies and colts had increasing trend of progesterone (Fig. 6.5B) and testosterone (Fig. 6.5C) levels similarly as the Miyazaki levels, respectively. However the levels in LS Miyazaki horses were significantly higher than the Hokkaido in March and February, respectively. Moreover, the expected first ovarian and testicular activities were found at late February and around late January in fillies (Fig. 6.5B) and colts (Fig. 6.5C), respectively, of both Hokkaido and Miyazaki horses at the progesterone over 1 ng/ml and testosterone reached to or higher 0.5 ng/ml. For estradiol-17β, LS Miyazaki horses had higher estradiol levels than those of Hokkaido significantly in some periods (Fig. 6.5A). The circulating cortisol concentration in LS Hokkaido horses tended to have lower cortisol levels than the Miyazaki during February while higher in April (Fig. 6.4D).

6.4 Discussion

According to my results, even though most of growth parameters in Miyazaki tended to be a little higher than Hokkaido, the significant difference between the two places were

not clearly notable under light supplementation condition. The body weight and girth circumference in Miyazaki colts seemed to have low values when compared to Hokkaido horses. This probably caused from the lower values of LS Miyazaki groups at the beginning of experiment in random sample selection (see in Chapter 5). The increment percent, under light supplementation, still showed the dramatic decrease of growth parameters in Hokkaido horses during winter January, especially in body weight and girth increments, whereas the LS Miyazaki horses showed opposite. Finally, those increments in LS Hokkaido horses rapidly increased to the levels similar to the Miyazaki or were higher at the end of experiment. These results indicated that even though body physical growth in Hokkaido horses were retarded in severe cold winter, after acclimatization growth rate became accelerated to be equally as the Miyazaki horses.

Regarding to hair coat conditions, the previous results of hair coat scores in horses under natural condition (Chapter 4) revealed that Hokkaido horses had the lower hair coat scores than the Miyazaki significantly in April. This indicated that Hokkaido horses are still covered by winter coat due to severe cold weather and delay molting while those in Miyazaki horses had shed already. In contrast, under light supplementation the hair coat scores of Hokkaido horses was increased in April to the levels similar to Miyazaki horses. This suggested that Hokkaido horses under natural condition were inferior in hair coat conditions, but turned to be good appearance similarly as Miyazaki horses by using light supplementation.

In the present study, higher T4 concentrations were noted in both LS Hokkaido colts and fillies as same as under natural condition (see Chapter 4). The higher levels of T4 suggested that Hokkaido horses dwelled in the colder north had basal level of thyroid hormone higher than Miyazaki horses raised in the south, due to the response to lower

ambient temperature for maintenance of body homeostasis in long term survival instead of growing. However, this higher T4 levels in Hokkaido horses were not consistent with the growth profile clearly. Previous publication reported that TRH administration resulted in the increase of plasma PRL and TSH levels, contrary to the result in TRH pre-treated mares followed by mixture of dopamine antagonist and growth hormone analog, which showed greater PRL response, with lower TSH response than mares without TRH (Gentry et al. 2002). Another study revealed that thyroxine injection as pre-treatment followed by exercise, $PGF_{2\alpha}$, or dopamine antagonist administrations had no effect on PRL responses in gelding horses (Thompson et al 2013). The present study showed that PRL concentration in LS Hokkaido horses increased to be equally to the Miyazaki and the increasing of PRL levels in light supplementation were not accompanied with T4 levels. Also, there was no significant correlation between PRL and T4 concentrations (data not shown). These results in conjunction with those previous studies suggested that circulating PRL elevation resulted from induction of artificial light supplementation via retina through the pineal gland and hypothalamic-anterior pituitary gland cascade, rather than the involvement of TRH stimulation. TRH might not act as a mediator in the response of PRL in horses.

Regarding the reproductive endocrine changes, estradiol-17β, progesterone and testosterone are associated with gonadal function in fillies and colts, respectively. This study found that progesterone and testosterone concentrations in LS Hokkaido groups were quite similar to the Miyazaki. However, some period showed significant higher levels in Miyazaki horses. This suggested that the higher levels of progesterone and testosterone in Miyazaki horses probably derived from the larger number of Miyazaki fillies and colts which had expected ovulation and active testicular function during blood collection time compared to the Hokkaido horses. Furthermore, LS Hokkaido fillies and colts seemed to have first ovarian

(late February) and testicular (around late January) activities in the same periods as the Miyazaki at the levels of progesterone over 1 ng/ml and testosterone equal to 0.5 ng/ml or higher, respectively. These indicated that the gonadal functions in Hokkaido yearlings under natural condition seemed to be slower in development when compared to the Miyazaki but showed improvement by light supplementation to be active similarly as the Miyazaki horses.

In conclusion, the present study clarified that under natural condition Hokkaido horses raised in the north seemed to be inferior to Miyazaki horses (south). However, providing of artificial light supplementation may help to improve body physical growth and early development of reproductive functions in Hokkaido yearlings to be as equal as Miyazaki horses. For further study, the total and free T3, free T4, TSH, basal and field metabolic rates should be investigated for more understanding in growth and metabolism in young horses.

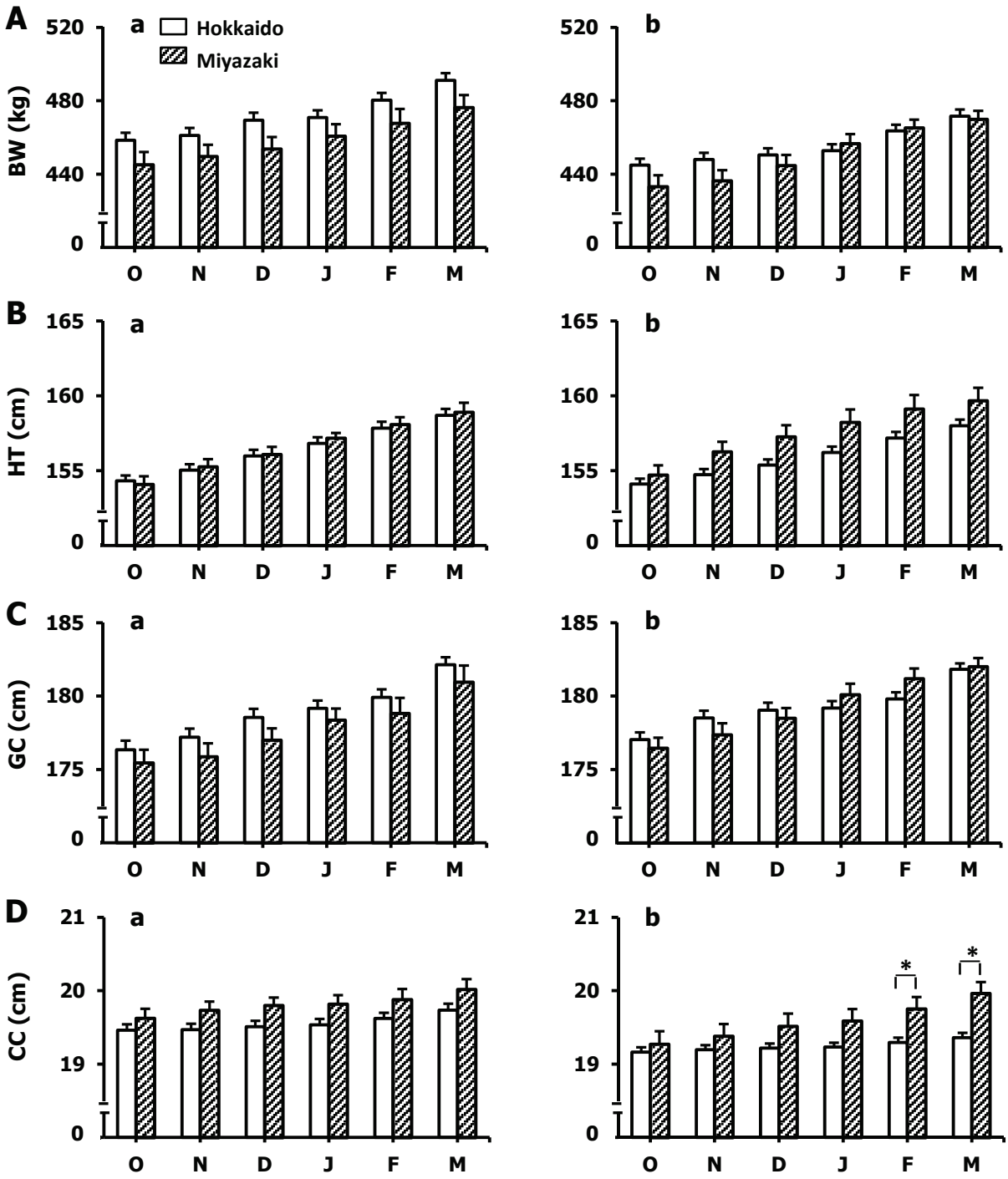


Fig. 6.1 Means of (A) body weight (BW), (B) height (HT), (C) girth (GC) and (D) cannon bone (CC) circumferences compared between Hokkaido (□) and Miyazaki (▨) in colts (a) and fillies (b) under light supplementation from October to March. Each value is expressed as the mean ± SEM. * Denoted the significant differences between different groups in each period and sex (P<0.05).

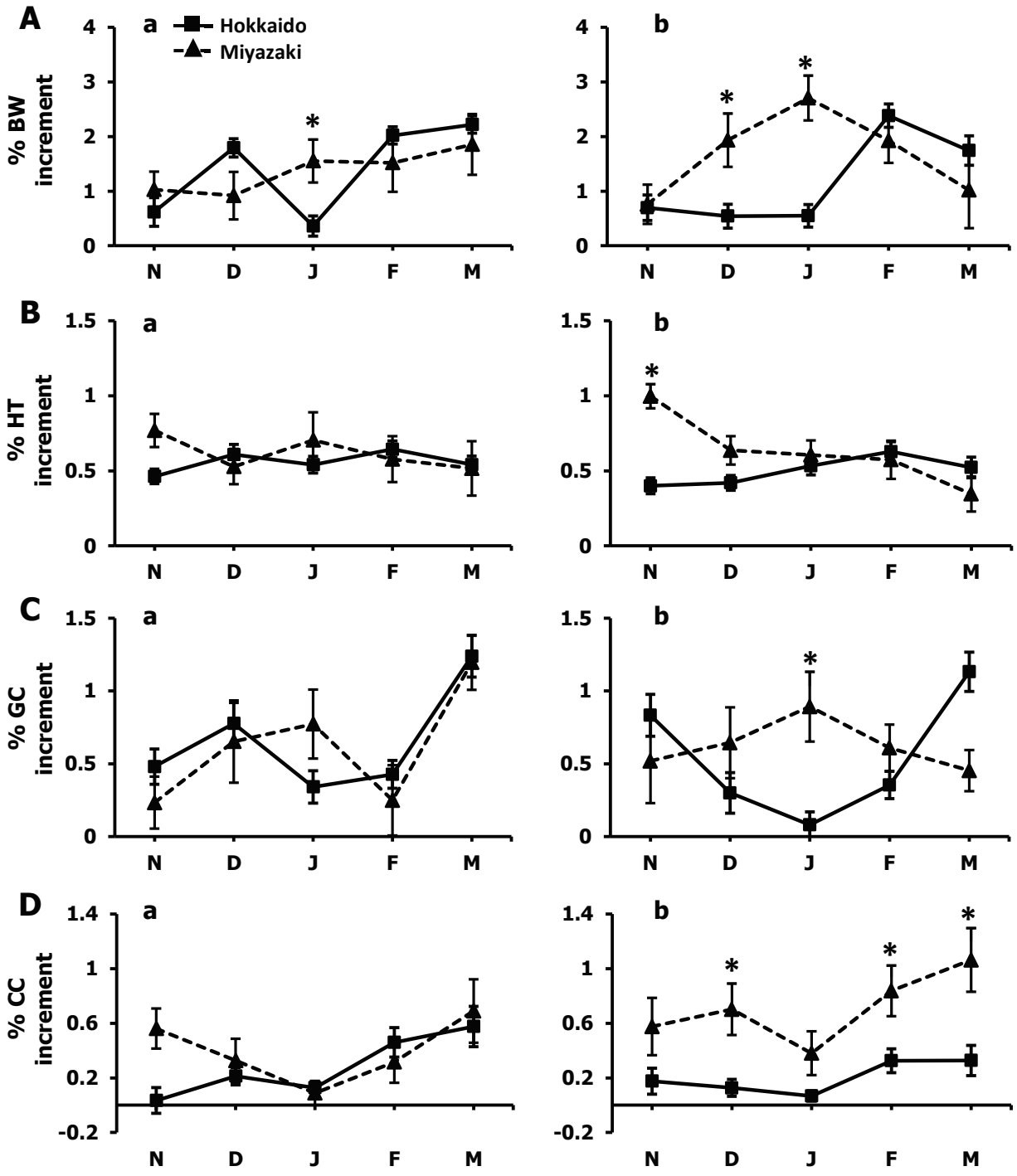


Fig. 6.2 Increment percent of (A) body weight (BW), (B) height (HT), (C) girth (GC) and (D) cannon bone (CC) circumferences compared between Hokkaido (–■–) and Miyazaki (--▲--) in colts (a) and fillies (b) under light supplementation from November to March. Each value is expressed as the mean ± SEM. * Denoted the significant differences between different groups in each period and sex (P<0.05).

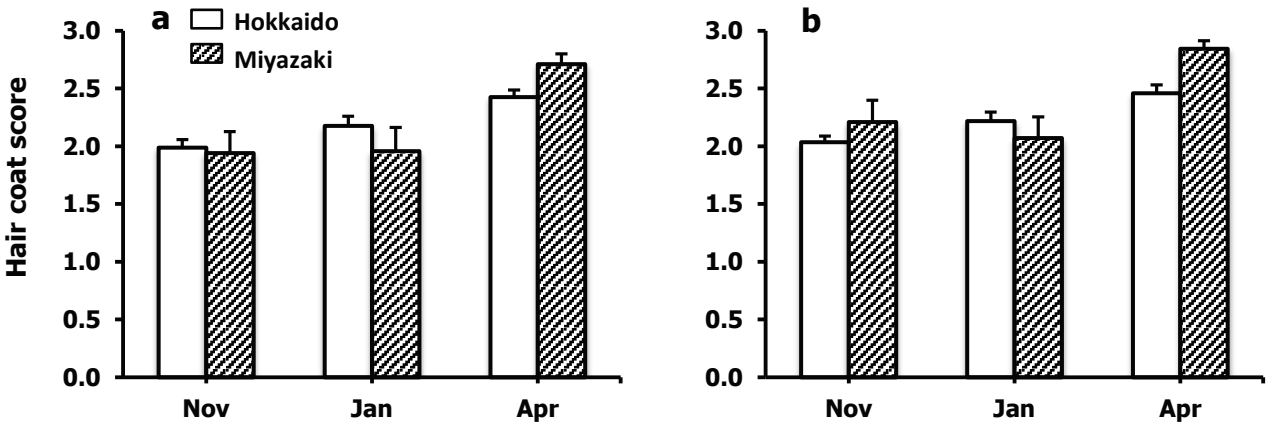


Fig. 6.3 Comparison of hair coat scores between Hokkaido (□) and Miyazaki (▨) in colts (a) and fillies (b) at November, January and April under light supplementation. Each value is expressed as the mean ± SEM.

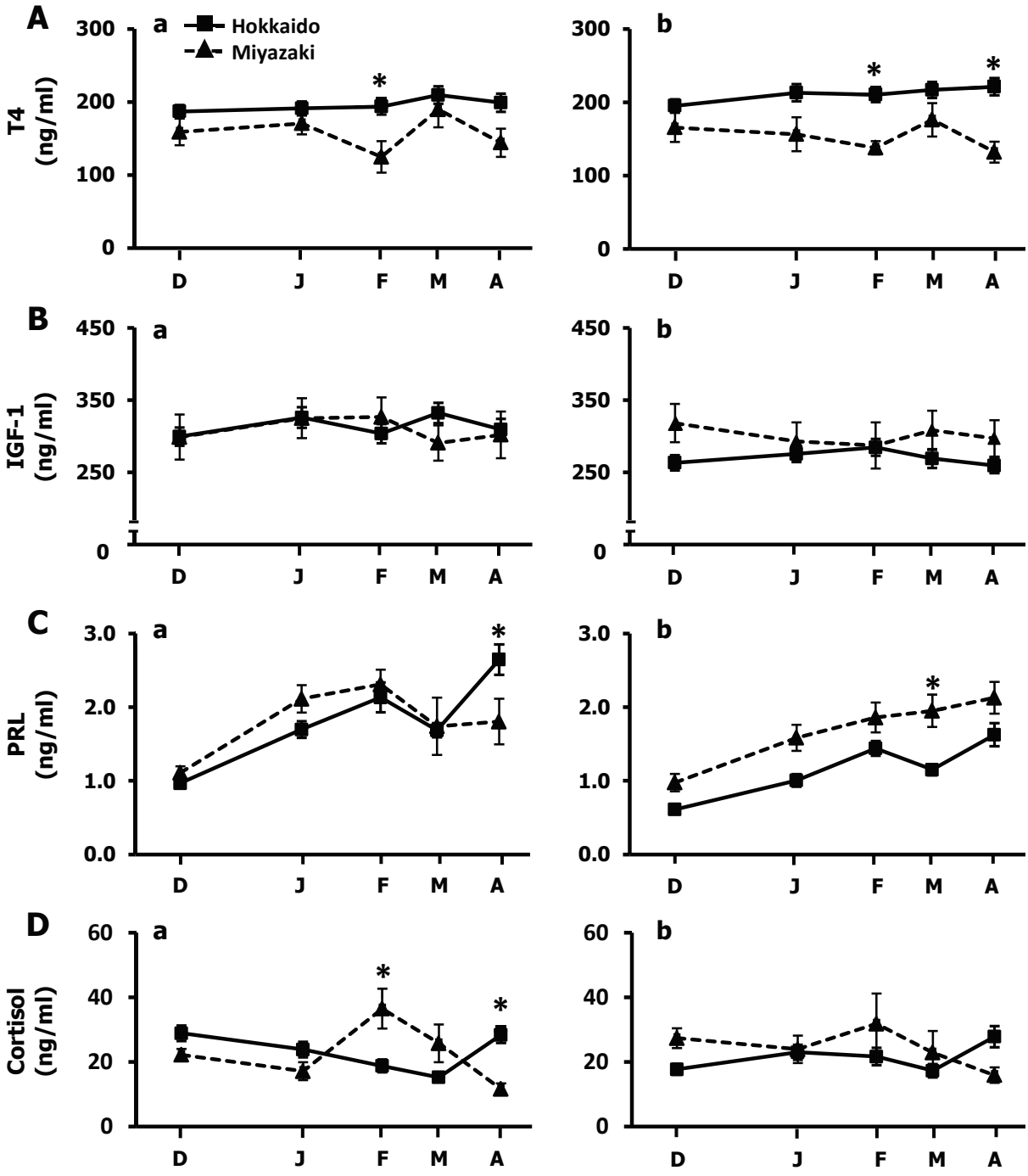


Fig. 6.4 Comparison of circulating T4 (A), IGF-1 (B), PRL (C) and Cortisol (D) concentrations (ng/ml) between Hokkaido (-■-) and Miyazaki (--▲-) in both colts (a) and fillies (b) under light supplementation from December to April. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in each period and sex (P<0.05).

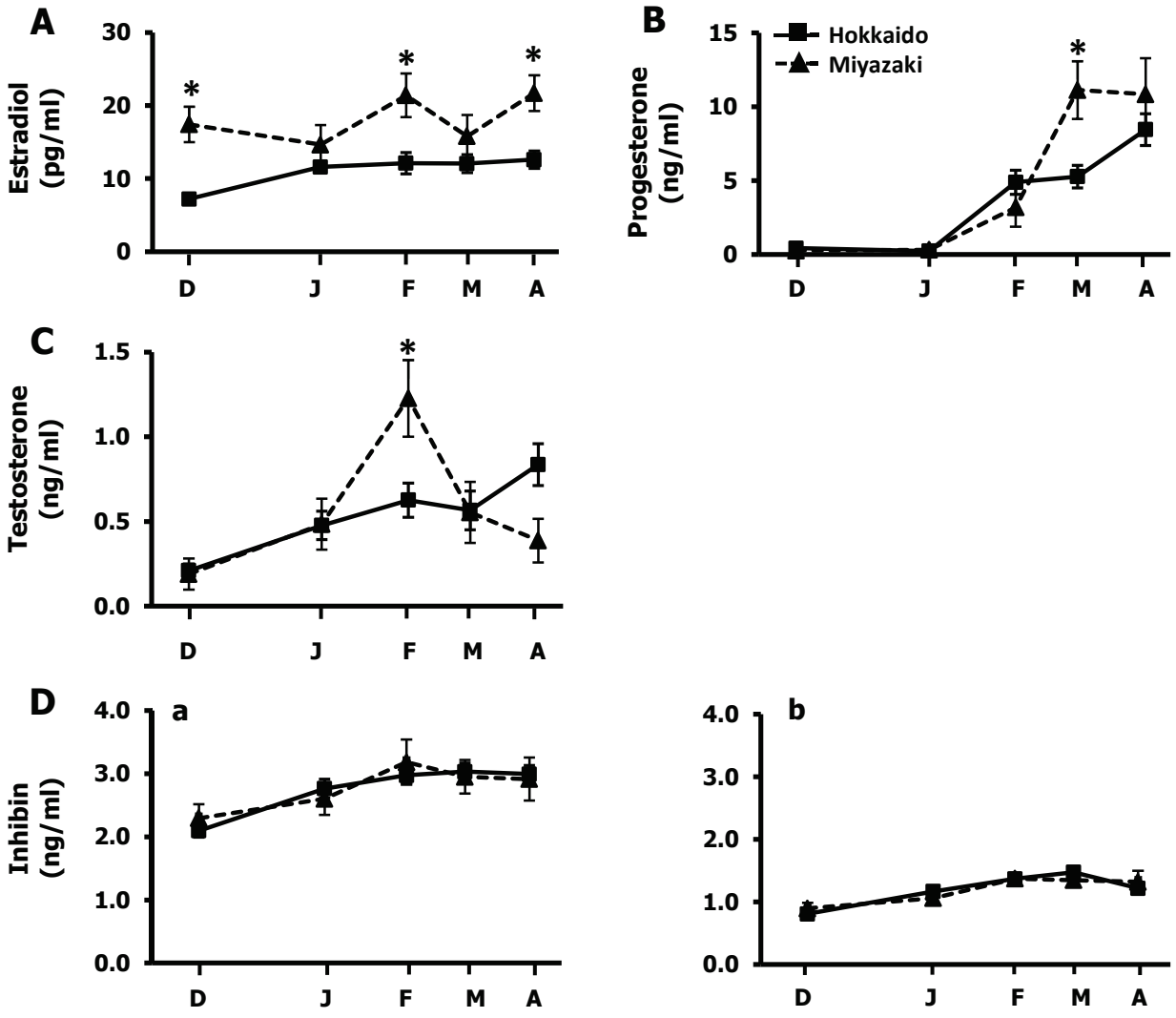


Fig. 6.5 Comparison of circulating concentrations of estradiol-17β (A) and progesterone (B) in fillies, testosterone (C) in colts and ir-inhibin (D) in both colts (a) and fillies (b) between Hokkaido (-■-) and Miyazaki (--▲--) under light supplementation from December to April. Values are expressed as mean ± SEM. * Denotes the significant differences between different groups in each period and sex (P<0.05).

Chapter 7

General discussion

People use horses for several purposes such as culture, work, therapeutics, entertainment, companionship and sport. In horse racing, racehorse is expected to have good conformations for high athletic performances. Therefore, the understanding of growth and development in yearling horses is important for equine husbandry to accomplish the target goals. Growth and development in animals are influenced by two major factors; namely, intrinsic and extrinsic factors. Intrinsic factors result from heredity mainly, and the extrinsic are referred as the environment factors acting from outside. These two kinds of factors are impossible to separate their specific influences, because they are interrelated each other entirely.

Environmental factors influence on the development process through learning and adaptation of animals. Climates, especially temperature and duration of sunlight or photoperiod, are crucial exogenous factors affecting humans and animals including horses in many ways. Horses are endotherms which maintain their own internal body temperature constantly by metabolic heat production. Changes of environmental temperature stimulate thermoregulation system to keep internal body temperature in equilibrium (homeostasis). In case of excessive cold as winter season, horses increase heat production and reduce heat loss. Thermoreceptor in the skins perceiving low ambient temperature relays the impulse to thermoregulating center in hypothalamus of the brain. Then, the signals from hypothalamus stimulate motor neuron resulting in increase of muscle metabolism and increase in metabolic heat production subsequently (Van Miert 2004). In long run of cold exposure,

maintenance of heat production without muscular heat production is driven by endogenous neurohormonal activity. In response to long-term external cold stimuli, either sympathetic nervous system activity or thyroid hormone secretion increases (Van Miert 2004). Consequently, shivering to increase body heat production, vasoconstriction of peripheral vessels (extremities, ear, muzzle) to reduce internal heat loss, and piloerection to trap warm surrounding air will occur (Blaxter 1989; Palmer 1983) (Fig. 7.1). The increase of thyroid hormone secretion to increase metabolic heat was found in my study. Yearlings in Hokkaido (severe cold) had higher T4 levels than the Miyazaki (less cold) through the winter until early spring. Previous studies also described that T4 concentrations were higher in colder weather comparing to warmer season (Brinkmann et al. 2016) and that T4 secretion was elevated during cold exposure in adult horses (Irvine 1967; McBride et al. 1985). T4 increase in cold weather indicated that horses dwelled in the colder north had higher basal metabolic rate than horses raised in the milder south. Nevertheless, my results showed that body growth and reproductive development were slower in Hokkaido horses under natural condition, especially in hard winter January, which was not coherent to those higher T4 level. Hokkaido horses with higher T4 level may adapt for survival but not enough for normal or rapid growth, contrary to Miyazaki. The lowest temperature in Miyazaki was higher than 0°C which is the lower critical temperature (LCT), the temperature at which metabolic heat production needs to increase for body core temperature maintenance, in limit-fed yearlings for normal growth (Cymbaluk 1994; Cymbaluk 1990). Miyazaki horses were able to keep growing through the periods and reaching the maximum limit, whereas Hokkaido yearlings had growth retardation during severe cold weather. However, after acclimatization, the reproductive function and body development of Hokkaido horses returned to be in the normal process with the increasing rate of growth parameters and hormonal concentration,

when enter early spring (March and April) (Fig. 4.5, 4.7-4.8). Moreover, horses respond to cold weather with behavioral changes, for example, seeking warm shelter, altering body posture and huddling (Duncan 1985). Hair coat turns to be thicker and longer to insulate from cold air and wind (Young and Coote 1973). Consistent with previous study (Kunii et al. 2015), my results showed that hair coat conditions in Hokkaido horses were thicker and longer than the Miyazaki in early spring (Fig. 4.6).

Natural photoperiod, another environmental factor influences on many kinds of body functions in humans, animals and plants. Circadian rhythm is the approximate 24-hour rhythms (light-dark cycle) driven by circadian clock which has been widely observed in plants, animals and other organism (Edgar et al. 2012). Animals adapt to rhythmic environmental changes such as the light-dark and seasonal cycles using their circadian clock which is located in suprachiasmatic nuclei (SCN) of the hypothalamus. Horses are known as typical long-day breeder with active reproductive functions during spring and summer by regulation of pineal gland and hypothalamic-pituitary-gonadal (HPG) axis. Photosensitive ganglion cells in the retina of the eyes receive light and send the signals to SCN via retinohypothalamic tract (Richardson 2005; Perreau-Lenz et al. 2004). Then the signals are passed to pineal gland. Melatonin secretion from pineal gland peaks at night then gradually decreases until it stops releasing in the morning (Vaughan et al. 1976). Duration of melatonin secretion is directly proportional to the length of the night. GnRH stimulates FSH and LH secretions from anterior pituitary gland resulting in activating gonadal functions and high estrogen and testosterone levels in circulation, respectively (Fig. 7.1). Signals via retinohypothalamic tract also synchronize circadian rhythm of growth hormone, prolactin, and cortisol (Lubkin et al. 2002).

As described above, neurohormonal activities as individual endogenous factors are stimulated and controlled by external environment stimuli. Growth hormone (GH) is

secreted by pituitary gland with regulation of growth hormone-releasing hormone (GHRH) and growth hormone-inhibiting hormone (GHIH) from hypothalamus (Amann 1993). GH stimulates growth, cell reproduction and cell regeneration in humans and other animals. GH also stimulates production of IGF-1 from liver. IGF-1 is involved in body growth (Fabian et al. 2004; Wang et al. 2009) and steroidogenesis (Gelber et al. 1992; Wang and Hardy 2004; Bernier et al. 1986; Perrard-Sapori et al. 1987). My study illustrated that IGF-1 and T4 levels in both Hokkaido and Miyazaki did not change clearly by light supplementation compared to natural condition. Photoperiod influences seemed to be limited in affecting IGF-1 and T4 secretions in yearling horses. In contrast, PRL results of my study in light supplementation showed increasing of PRL levels in both Hokkaido and Miyazaki consistent with previous reports (Nambo et al. 2010, Kunii et al. 2015). The increased PRL concentrations, accordant with the light supplementation improved hair coat condition and advanced molting in yearlings, as consistently with previous studies (Nishikawa 1959; Kooistra and Ginther 1975; Numbo et al. 2010, Kunii et al. 2015). Furthermore, my results consistent with previous publications (Kunii et al. 2015, Suzuki et al. 2015), showed the increase of growth parameters, earlier development of first ovarian and testicular activities in young horses by light supplementation. These suggested that the light-induced prolactin increase might hasten gonadal function in yearlings by the increase in number of LH receptor in granulosa/theca, CL of ovary (Jones and Hsueh 1981; Van Straalen and Zeilmaker 1982) and Leydig cells of testis (Hair et al. 2002, Henderson et al. 2006, Daoud and Ezzo 2014). In addition, PRL is reported to be indirectly involved in growth and development by promoting calcium absorption in intestine (Charoenphandhu et al. 2009 and 2010; Suntornsaratoon et al. 2010), and expressing of PRL receptor in epiphyseal plate (Suntornsaratoon et al. 2010). However, my result found that increasing of PRL levels due to light effect did not conform to

T4 increase. No correlation in PRL and T4 secretion in my study were consistent with previous studies, reporting that TRH pre-treated mares followed by combination of dopamine antagonist and growth hormone analog, showed greater PRL response but lower TSH response than mares without TRH (Gentry et al. 2002). Another study revealed that pre-treatment by thyroxine followed by exercise, $PGF_{2\alpha}$, or dopamine antagonist administration did not affect to prolactin responses in gelding horses (Thompson et al. 2003). In conjunction with previous report, my results suggested that the elevation of prolactin level caused by stimulation of light supplementation through the eye, pineal gland and hypothalamic-pituitary gland cascade, rather than the stimulation of TRH. Therefore, TRH might not act as a mediator in the response of prolactin to light supplementation in yearlings.

In conclusion, both external environmental and internal neuroendocrine factors play important role in regulation of growth and development in yearling horses. High T4 levels of yearling horses in the cold weather may be essential for adapting and maintaining body condition to survive. However, light supplementation can enhance PRL secretion and improve hair coat shedding and stimulate gonadal activity in yearling horses.

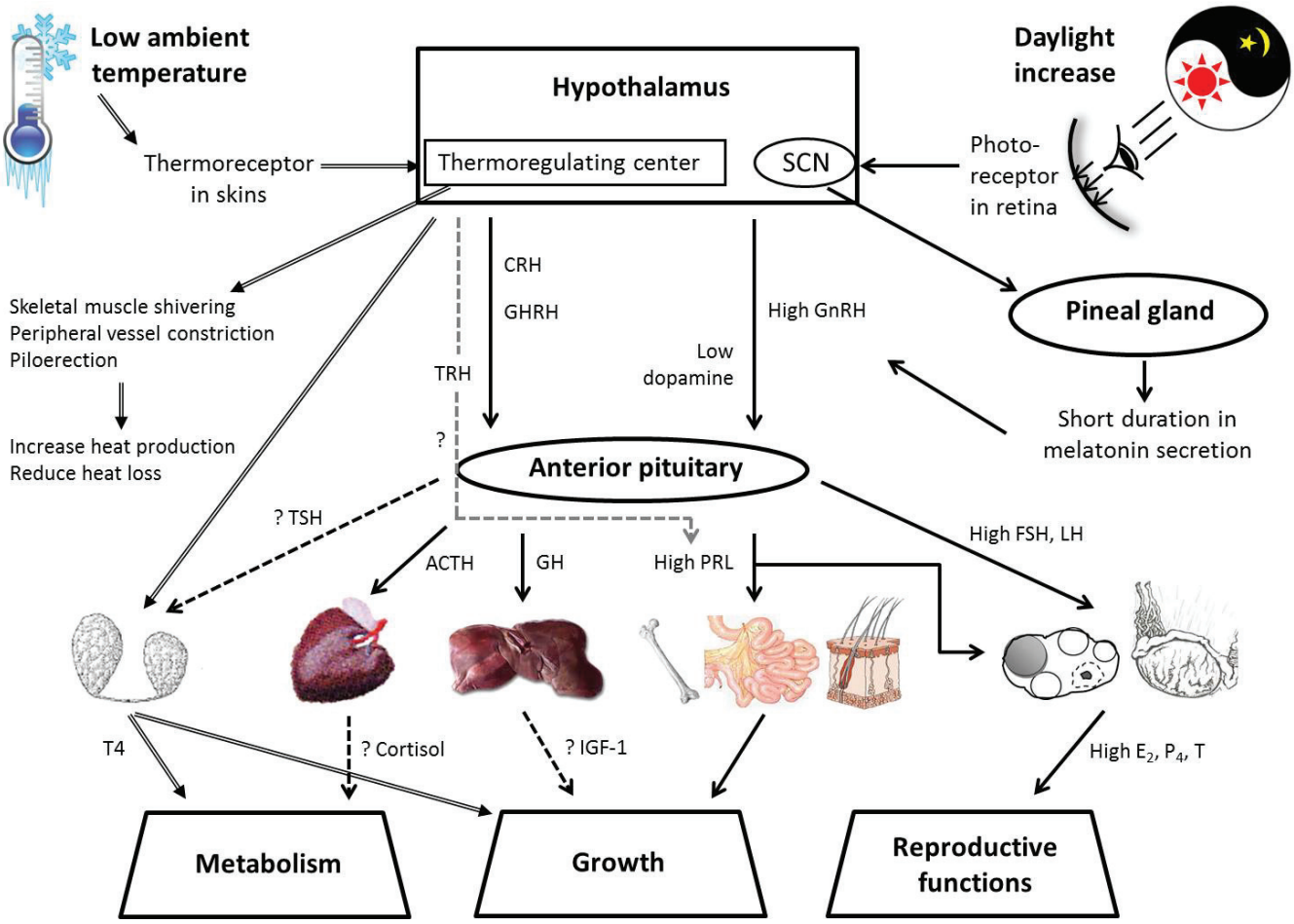


Fig. 7.1 Schematic diagram for summary of possible pathway is associated with temperature and photoperiod effects on growth and reproductive functions in yearling horses.

Chapter 8

Summary

8.1 Establishment of a novel radioimmunoassay system for total thyroxine (T4) measurement in horse

I attempted to establish a novel radioimmunoassay system which was more suitable and reliable for circulating total T4 measurement in horses. During decades, commercial immunoassay kits which were developed for human samples have been widely applied to measure T4 concentration in horses because the simplicity of measurement procedure. Plasma proteins which bind to T4 in blood circulation have species variation in both quantity and binding affinity. These differences among species influence on accurateness in circulating T4 detection by using commercial kits which have been produced specifically for human. Therefore, it is essential to separate T4 from binding proteins and optimize the assay protocol to determine true value of circulating T4 in horse.

Radioimmunoassay (RIA) has been regarded as the gold standard method for thyroid hormone measurement due to the advantage of high sensitivity, specificity, and low detection limits. Equilibrium dialysis and ultrafiltration are commonly used for dissociate binding proteins in thyroid hormone measurement however these techniques are long time-consuming for lots of samples determination. According to the sodium salicylate method which had been used to separate binding proteins before RIA for thyroid hormone measurement in rat, I applied this method to measure T4 concentration in horse blood samples. However, my results showed that the use of original 2% sodium salicylate markedly

affected the binding of T4 radioligand to the first antibody. Furthermore, my further trial found that sodium salicylate at 0.01% had no effect on tracer-antibody binding. Consequently, the dilution to 0.01% made T4 levels too low to detect in horse serum samples. For this reason, I looked for alternative method instead of sodium salicylate for binding protein separation prior to RIA in horse blood samples.

I chose protein separation protocol for IGF-1 measurement, the acid ethanol method, to apply in T4 determination, since the acid ethanol protocol is well known and commonly used for protein separation. The modified acid ethanol protocol revealed new levels of circulating T4 in horse plasma samples higher than previous report however it required proceeding in several steps and also was more time-consuming. From these reason, I tried further to find a simpler protocol using sodium acetate ethanol method. This technique is popular for protein, DNA and RNA precipitation using alcohol and sodium acetate. Modified sodium acetate ethanol method protocol had the capability to separate bound proteins from T4 appropriately and had no differences in T4 concentrations from modified acid ethanol method of IGF-1. These indicated that use of the modified sodium acetate ethanol method not only possessed effectiveness in binding protein removal similar to acid ethanol but also simpler.

As the validation of the RIA with respect to modified sodium acetate ethanol method by comparing T4 concentrations between yearling and adult horses, the results demonstrated that yearlings (209.17 ± 15.05 ng/ml) had T4 concentrations higher than pervious reported values in yearling horses and also 6-fold higher than in diestrous mares (34.92 ± 13.69 ng/ml) approximately. This indicated that my assay protocol was reasonable to detect the physiological differences between different age and development conditions. In addition, I compared circulating T4 levels in yearling horses raised in different climatic

conditions in Japan. This comparison was conducted by using Hokkaido horses in the north the temperate zone had lower temperatures throughout the experimental periods than Miyazaki horses in the subtropical climate of the south. My result revealed that circulating T4 concentrations in Hokkaido yearlings tended to be higher than in Miyazaki horses throughout these periods. This indicated that my assay was appropriate to investigate the physiologic responses at different climates. All of those results suggested that my immunoassay seemed to be more physiological and appropriate in separating of binding protein and revealing the true value of total T4 in equine species.

8.2. Comparison of metabolic and reproductive endocrine functions between north and south climates of Japan in Thoroughbred trained yearling horses under natural and light supplementation conditions

This study aimed to compare body growth, metabolic, and reproductive hormonal changes in Thoroughbred trained yearling horses under different climate conditions with/without artificial light supplementation (LS). Total 160 Thoroughbred trained yearlings, which were subjected to the study from 1 year of age at September in autumn to less than 2 year of age at April in spring, raised in two facilities of the Japan Racing Association (JRA); Hidaka Training and Research Center in Hokkaido (temperate north, latitude 42.2° and longitude 142.8°) or Miyazaki Training Yearling Farm in Miyazaki (subtropical south, latitude 31.9° and longitude 131.4°). All horses were divided into the control and the light supplementation groups. Ninety-one Hokkaido (44 colts, 47 fillies) and 22 Miyazaki (11 colts, 11 fillies) yearlings were exposed LS, 2 times per day to create extended photoperiod of 14.5 h daylight and 9.5 h of dark time, equal to summer from December 25th to April 16th in each

year (2012-2013, 2013-2014) by using timer-linked 100 watt white light bulb. The light was set in the ceiling of the horse stall. The horses in control groups exposed to natural light only and had not receivd LS throughout experimental periods. Regarding growth, the body weight, height, girth and cannon bone circumferences were measured monthly. Hair coat condition was scored by inspection. Circulating total thyroxine (T4), Insulin-like growth factor-I (IGF-1), prolactin (PRL), ir-inhibin and cortisol concentrations were measured by radioimmunoassay (RIA). The circulating sex steroid hormones, estradiol-17β (E_2), progesterone (P_4), testosterone (T), concentrations were determined by Time-resolved fluoroimmunoassay commercial kits.

Under natural condition, all of growth parameters in Hokkaido yearlings seemed to be lower than Miyazaki horses. Also, hair coat scores were lower in Hokkaido yearlings significantly when compared to the Miyazaki. Circulating T4 concentrations of Hokkaido yearlings tended to be higher than those of Miyazaki. In contrast, IGF-1 (colt), PRL (colt and filly) and E_2 (filly) levels in Hokkaido were significantly lower than the Miyazaki. These result indicated that differences in climates between the north and south of Japan affected to growth and early reproductive development in young horses. In addition, Hokkaido yearlings had slower growth rate and early gonadal functions than Miyazaki horses.

In light supplementation, the body weight and girth increment percent of Hokkaido yearlings in January dramatically decreased and then eventually increased similarly as Miyazaki levels during February and March. Hair coat scores of LS Hokkaido yearlings also reached to the levels of Miyazaki at the end of experiment time. Circulating PRL and P_4 concentrations in both LS Hokkaido and Miyazaki groups were higher than the controls significantly and showed similar levels between LS Hokkaido and Miyazaki groups. Expected first ovarian (late February) and testicular (late January) activities in both LS Hokkaido and

Miyazaki groups were seemed to be earlier than the controls (early of April and late of February, respectively). Furthermore, no differences in periods of the first ovarian and testicular activity detection were noted between LS Hokkaido and Miyazaki horses. For hair coat scores at April, the scores of LS Hokkaido yearlings increased to be higher than the controls significantly and reach similarly to Miyazaki levels. The PRL consistent with P_4 and T concentrations in LS Hokkaido yearlings increased and reached to the similar level as Miyazaki. All of my results were clarified that under naturally colder condition Hokkaido horses exhibited higher basal metabolism to maintain homeostasis for survival and seemed to be inferior to Miyazaki yearlings. However, providing of LS may help to improve growth and early development of reproductive function in Hokkaido yearlings to be as equal as Miyazaki horses.

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