สัณฐานวิทยาของเซลล์เม็ดเลือดและพารามิเตอร์ทางโลหิตวิทยาของกบนา *Hoplobatrachus rugulosus* (Wiegmann, 1834)

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BLOOD CELL MORPHOLOGY AND HEMATOLOGICAL PARAMETERS OF RICE FIELD FROG *Hoplobatrachus rugulosus* (Wiegmann, 1834)

Mr. Suthirote Meesawat

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By	Mr. Suthirote Meesawat
Field of Study	Zoology
Thesis Advisor	Jirarach Kitana, Ph.D.
Thesis Co-Advisor	Noppadon Kitana, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate ProfessorPolkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Duangkhae Sitthicharoenchai, Ph.D.)

.....Thesis Advisor
(Jirarach Kitana, Ph.D.)

.....Thesis Co-Advisor
(Noppadon Kitana, Ph.D.)

.....Examiner
(Assistant ProfessorWichase Khonsue, Ph.D.)

.....External Examiner
(Associate ProfessorPutsatee Pariyanonth)

สุธิโรจน์ มีสวัสดิ์ : สัณฐานวิทยาของเซลล์เม็ดเลือดและพารามิเตอร์ทางโลหิตวิทยาของกบนา *Hoplobatrachus rugulosus* (Wiegmann, 1834) (BLOOD CELL MORPHOLOGY AND HEMATOLOGICAL PARAMETERS OF RICE FIELD FROG *Hoplobatrachus rugulosus* (Wiegmann, 1834)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.จิรารัช กิตนะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.นพดล กิตนะ, 144 หน้า.

กบนา *Hoplobatrachus rugulosus* เป็นกบที่พบทั่วไปตามพื้นที่ชุ่มน้ำในประเทศไทยและเป็นที่รู้จักดีในฐานะสัตว์เศรษฐกิจที่สำคัญ เทคนิคทางโลหิตวิทยาเป็นวิธีการพื้นฐานที่ใช้ในการตรวจสอบสุขภาพของสัตว์ แต่ข้อมูลทางโลหิตวิทยาซึ่งมีความสำคัญสำหรับการจัดการและการอนุรักษ์กบชนิดนี้ ยังมีอยู่จำกัดมาก กลุ่มวิจัยของเราจึงมุ่งเน้นที่จะพัฒนาเกณฑ์ในการประเมินสุขภาวะของกบนาในที่เลี้ยงโดยอาศัยพารามิเตอร์ทางโลหิตวิทยา ดังนั้นจึงดำเนินการศึกษาค่าทางโลหิตวิทยาและสัณฐานวิทยาของเซลล์เม็ดเลือดของ *H. rugulosus* ที่จับจากธรรมชาติและศึกษาค่าทางโลหิตวิทยาและค่าอ้างอิงปกติทางโลหิตวิทยาของ *H. rugulosus* ที่เลี้ยงในฟาร์ม โดยเก็บกบนาตัวเต็มวัยจากแหล่งอาศัยในธรรมชาติที่จังหวัดน่าน ในฤดูฝนปี พ.ศ. 2557 และเก็บกบนาพ่อแม่พันธุ์ กบนารุ่น และกบนาป่วย จากฟาร์มเลี้ยงกบในจังหวัดเชียงใหม่ ใน 3 ฤดูกาลของปี พ.ศ. 2557 แล้วเก็บตัวอย่างเลือดนำมานับจำนวนเซลล์เม็ดเลือดแดงและเซลล์เม็ดเลือดขาวโดยใช้ฮีโมไซโตมิเตอร์ หาค่าเซลล์เม็ดเลือดอัดแน่น (PCV) นับจำนวนเซลล์เม็ดเลือดขาวจากสไลด์เลือดที่ย้อมด้วยสีกิมซ่า และหาค่าโปรตีนรวมจากพลาสมา ผลการศึกษาในกบธรรมชาติ ทำให้สามารถจำแนกและทราบลักษณะทางสัณฐานของเซลล์เม็ดเลือดของกบชนิดนี้ ได้แก่ เซลล์เม็ดเลือดแดง เซลล์เม็ดเลือดแดงอ่อน ทรอมโบไซต์ เซลล์เม็ดเลือดขาวชนิดลิมโฟไซต์ โมโนไซต์ นิวโทรฟิล อีโอซิโนฟิล และเบโซฟิล ได้ค่าเฉลี่ย PCV และค่าเฉลี่ยสัดส่วนเซลล์เม็ดเลือดขาวชนิดลิมโฟไซต์ โมโนไซต์และนิวโทรฟิล ซึ่งพบว่ามีความแตกต่างระหว่างเพศอย่างมีนัยสำคัญทางสถิติ ส่วนผลการศึกษาในกบนาพ่อแม่พันธุ์ในฟาร์ม พบว่าค่าเฉลี่ยของจำนวนเซลล์เม็ดเลือดแดง PCV โปรตีนรวมในพลาสมา และสัดส่วนเซลล์เม็ดเลือดขาวชนิดลิมโฟไซต์ โมโนไซต์ และอีโอซิโนฟิล มีความแตกต่างระหว่างเพศอย่างมีนัยสำคัญทางสถิติ ส่วนในกบนารุ่นในฟาร์มเลี้ยง พบว่าค่าเฉลี่ยของ PCV โปรตีนรวมในพลาสมา และสัดส่วนเซลล์เม็ดเลือดขาวชนิดลิมโฟไซต์ โมโนไซต์ นิวโทรฟิล และอีโอซิโนฟิล มีความแตกต่างระหว่างเพศอย่างมีนัยสำคัญทางสถิติ นอกจากนี้ยังพบว่าค่าพารามิเตอร์ทางโลหิตวิทยาของกบนาพ่อแม่พันธุ์และกบนารุ่นในฟาร์มเลี้ยงส่วนใหญ่ มีความแตกต่างกันระหว่างฤดูกาลยกเว้นสัดส่วนเบโซฟิล เมื่อทดสอบแล้วพบว่าค่าอ้างอิงปกติทางโลหิตวิทยาของกบนาฟาร์มพ่อแม่พันธุ์ที่มีความไวต่อการใช้ตรวจสอบสุขภาวะของกบในฟาร์มเลี้ยงได้ คือ ค่าจำนวนเซลล์เม็ดเลือดแดง (สำหรับเพศเมียในฤดูฝน) ค่าโปรตีนรวมจากพลาสมา (สำหรับทั้งสองเพศในฤดูแล้งหนาว) และค่าจำนวนเซลล์เม็ดเลือดขาวชนิดลิมโฟไซต์ (สำหรับทั้งสองเพศในฤดูฝน) โดยสรุปค่าพารามิเตอร์ทางโลหิตวิทยาและค่าอ้างอิงปกติทางโลหิตวิทยาที่ได้จากการศึกษานี้ เป็นการรายงานครั้งแรกและจะเป็นข้อมูลพื้นฐานที่สำคัญที่จะนำไปใช้ในการศึกษาสุขภาวะเพื่อการอนุรักษ์และการจัดการประชากรกบนา *H. rugulosus* ทั้งในธรรมชาติ และฟาร์มเลี้ยงในประเทศไทย

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SUTHIROTE MEESAWAT: BLOOD CELL MORPHOLOGY AND HEMATOLOGICAL PARAMETERS OF RICE FIELD FROG *Hoplobatrachus rugulosus* (Wiegmann, 1834). ADVISOR: JIRARACH KITANA, Ph.D., CO-ADVISOR: NOPPADON KITANA, Ph.D., 144 pp.

The rice field frog *Hoplobatrachus rugulosus*, a common anuran species found in wetlands throughout Thailand, is well known as an economically important farm animal. Hematological technique is a basic method to determine health of animals. Even though the data on hematology is crucial for manipulation and conservation of this frog, they are still very limited. Our research group tries to develop health assessment criteria based on hematological parameters of the frog. Therefore, hematological parameters including blood cell morphology and morphometry of wild caught *H. rugulosus* and hematological parameters with the normal reference values of farm raised *H. rugulosus* were examined in this study. The adult wild frogs were collected from natural habitat at Nan Province in wet season, 2014. The farm raised frogs including adult, juvenile and sicked frogs were collected from farm in Chiang Mai Province in 3 seasons during 2014. Blood samples were subjected for erythrocyte and leukocyte count by hemocytometer, packed cell volume (PCV) estimation, differential leukocyte count from Giemsa-stained blood smears and total protein quantification by Bradford assay. The results showed the morphological characteristics of wild *H. rugulosus* blood cells including erythrocytes, immature erythrocytes, thrombocytes, lymphocytes, monocytes, neutrophils, basophils and eosinophils. In wild caught frogs, the mean of PCV, number of lymphocytes and neutrophils showed significant sex-related difference. The hematological parameters of farm raised adult *H. rugulosus* that showed significant sex-related differences include erythrocyte count, PCV, plasma total protein, differential lymphocyte count, differential monocyte count, and differential eosinophil count. The hematological parameters of farm raised juvenile frogs that showed significant sex-related differences include PCV, plasma total protein, differential lymphocyte count, differential monocyte count, differential neutrophil count and differential eosinophil count. Most of hematological parameters of farm raised adult and juvenile frogs also showed seasonal variation except the differential basophil count. The normal reference values that have been established and validated to be sensitively used for monitoring of health status of adult farm raised *H. rugulosus* include erythrocyte count (for female in wet season), plasma total protein (for both sexes in cool dry season) and differential lymphocyte count (for both sexes in wet season). The hematological parameters and the normal reference values presented in this study could be regarded as the first report and a crucial baseline data for conservation and management of wild and farm raised *H. rugulosus* in Thailand.

Department:	Biology	Student's Signature	.....
Field of Study:	Zoology	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

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CHAPTER I
INTRODUCTION

Blood is a specialized connective tissue mainly circulated in the circulatory system of vertebrates. Its many functions include transport of nutrients and oxygen to cells, transport of wastes and carbon dioxide away from cells, delivery of hormones and other substances to cells throughout the body, maintenance of homeostasis by acting as a buffer and by participating in coagulation and thermoregulation and transport cells of the immune system that protect the body from pathogenic agents (Ross and Pawlina, 2006; Young and Heath, 2000). Hematology is the study of morphology, physiology and pathology of blood. It contributes in some part to a success in diagnosis and treatment of diseases. Hematological study in a species always starts with a study of morphology of blood cells, then followed with other hematological aspects. Blood consists of two major components, the formed elements and the extracellular matrix called plasma. The formed elements of mammalian blood consist of blood cells and their derivatives including erythrocytes, leukocytes and platelets (Mescher, 2010). In non-mammalian vertebrates, the platelets are replaced by cells called thrombocytes whose function is also involved in blood clotting initiation process (Campbell, 2015).

Hematological parameters are measurable factors that have been used to determine various blood components. There are many recommended hematological parameters that suitable for health evaluation of non-mammalian and mammalian vertebrates including erythrocyte and leukocyte counts, packed cell volume, plasma total protein, total leukocyte count and differential leukocyte count. Changes in hematological parameters such as change in morphology of blood cells and change in number of different kinds of blood cells may related to the physiological status and can indicate the health status of the body at that time (Salakij, 2005). These parameters have been used with several wildlife species, especially with threatened or endangered populations, and may aid in evaluating ecosystem health (Arıkan and Cicek, 2010). The blood parameter value may change from the normal reference value if there is disturbance in the body. The normal reference value in hematology is

available in human and many domestic mammals. But, in non-mammalian vertebrates, especially amphibians, it is still very limited (Allender and Fry, 2008)

Amphibians are known as sensitive animals. Amphibian blood responses to protect them against pathogens and environmental stresses, which may disturb their physiology, psychology, growth and breeding (Burgmeier et al., 2011; Davis et al., 2008). Some previous studies reported the characteristics of amphibian blood (Bricker et al., 2012; Canfield, 1998; Claver and Quaglia, 2009; Das and Mahapatra, 2012). Most of them studied on hematology in various species of *Rana* (Davis et al., 2008; Omonona and Ekpenko, 2011; Palenske and Saunders, 2003; Sinha, 1983; Weathers, 1975). In this study, the rice field frog *Hoplobatrachus rugulosus* (Wiegmann, 1834) is an interested species. It is an anuran species found in wetlands throughout Thailand (Amatyakul, 1995). This frog is commonly been consumed as food by local people. It also has the potential to be used as a model for research in many fields, such as physiology, reproductive biology and ecotoxicology (Ratanasaenga et al., 2008; Ruamthum et al., 2011). But, their basic data of hematology is very scarce. Currently, the wild *H. rugulosus* population is decreasing due to habitat loss and interference from human activities. However, it is still been found in some areas in Thailand especialy in several northern provinces including Nan Province, which contain many wetland agricultural areas (Pansook, 2010). These agricultural areas are still a suitable habitat for *H. rugulosus*, so it is possible to obtain specimens from natural habitat.

As it is well known that this frog is an economically important animal of Thailand, *H. rugulosus* farming is increasing and becomes more popular in order to serve market demand. However, there is lack of standard system to cultivate the frog in farm. Like other farmed animals, farmed frogs often have stress and diseases due to limitation of space. Currently, the health status monitoring of farmed frogs is generally based on visual inspection because there is no report about normal reference value of hematological parameters of this frog that can be used to assess the health status of the frogs and monitor the spread of disease in the ponds. Therefore, the hematological parameters and hematological normal reference values that can effectively be used to evaluate the health status would reduce the risk of disease spreading in the frog farming.

Objectives

1. To investigate blood cell morphological characters of the rice field frog *H. rugulosus*
2. To determine hematological parameters of the rice field frog *H. rugulosus* in nature
3. To determine normal reference values of hematological parameters of the rice field frog *H. rugulosus* in farm system

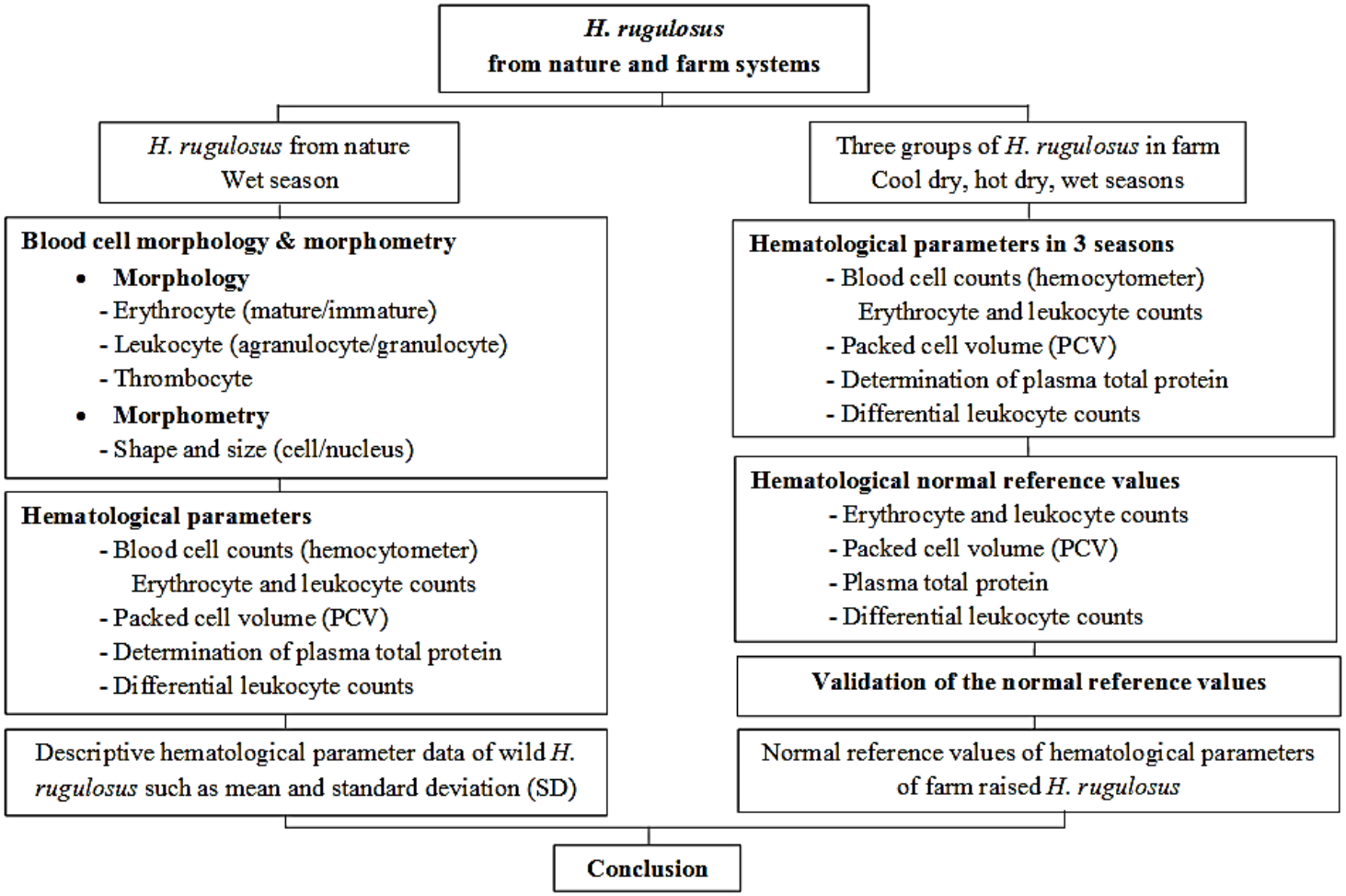

Figure 1-1 Research scope of this study

CHAPTER II
LITERATURE REVIEWS

1. Hematology

Blood is a specialized connective tissue in circulatory system of animals. Blood consists of formed elements and extracellular matrix whose volume exceeds that of the formed elements. In vertebrates, blood is propelled through the cardiovascular system by the pumping action of the heart to reach the body tissues (Campbell and Reece, 2004; Mescher, 2010; Ross and Pawlina, 2006). Its many functions include transport of nutrients and oxygen to cells, transport of wastes and carbon dioxide away from cells, delivery of hormones and other substances to cells throughout the body, maintenance of homeostasis by acting as a buffer and by participating in coagulation and thermoregulation and transport cells of the immune system that protect the body from pathogenic agents (Ross and Pawlina, 2006; Young and Heath, 2000).

Hematology is the study of morphology, physiology and pathology of blood. It contributes in some part to the success in diagnosis and treatment of diseases. Hematological study in a species commonly starts with a study of morphology of blood cells, and then followed with other hematological aspects, involving both the formed element part and the extracellular matrix part. The formed elements of mammalian blood consist of blood cells and their derivatives including erythrocytes, leukocytes and platelets (Mescher, 2010). In non-mammalian vertebrates, the platelets are replaced by cells called thrombocytes (Campbell, 2015).

Erythrocytes are the most numerous blood cells. The main function of erythrocytes is oxygen-carrying. The erythrocyte cytoplasm is filled with protein called hemoglobin that functions in oxygen-binding and oxygen-carrying. Leukocytes are classified into two groups based on the presence or absence of prominent specific granules in the cytoplasm. The leukocytes with specific granules are classified as granulocytes, including neutrophils, eosinophils and basophils. The leukocytes without specific granules are classified as agranulocytes, including lymphocytes and monocytes. Their collective function is to fight infections. Monocytes and neutrophils

are phagocytes, which engulf and digest bacteria and debris from the body's own dead cells. Lymphocytes develop into specialized B cells and T cells, which produce the immune response against foreign substances. Eosinophils defend against parasites and moderate the inflammatory processes. Basophils release of histamine and other inflammatory mediators and develop into precursors of mast cells in peripheral tissues (Ross and Pawlina, 2006). Blood platelets or thrombocytes promote blood clotting and help repair minor tears or leaks in the wall of blood vessels, preventing loss of blood (Mescher, 2010). Abnormal morphology of blood cells and change in number of different kinds of blood cells may related to the physiological status and may indicate the health status of the body at that time (Bain et al., 2012; Salakij, 2005).

Blood plasma is fluid extracellular material in which the formed elements are suspended. The plasma volume in whole blood is approximately 55%. More than 90% by weight of plasma is water, which is the solvent for a variety of solutes, including proteins (albumin, globulins and fibrinogen), dissolved gases, electrolytes, nutrients, regulatory substances and waste materials. The solutes in the plasma help maintain homeostasis (Mescher, 2010; Ross and Pawlina, 2006). Keeping both the components and the amount of blood at constant level is essential for the continuity of life. Plasma parameter change such as change in plasma protein content may relate to physiological imbalance and may indicate the health status of the body (Bain et al., 2012; Salakij, 2005).

Hematological technique is a basic method to determine health of the animal. It is a useful tool for diagnosis and health monitoring for vertebrates (Campbell, 2004; Canfield, 1998; Salakij, 2005). Because this methodology is relatively rapid and easy compared with other methods (Salakij, 2005), the result from hematological study is frequently used to indicate the basic animal health status. It has been reported that changes of hematological parameters can indicate stress (Davis et al., 2008) and inception of disease in animals (Burgmeier et al., 2011). Hematological parameters that frequently have been used to assess the health of animals are packed cell volume or hematocrit, erythrocyte count, leukocyte count and differential leukocyte count. These parameters are important fundamental to track the health of the animals (Salakij, 2005).

Packed cell volume (PCV) is a measure of the ratio of the volume occupied by erythrocytes to the volume of whole blood in a blood sample. The ratio is measured after an appropriate centrifugation (Bain et al., 2012). It is usually expressed either as a percentage or as a decimal fraction (e.g. 35% or 0.35). The PCV value has been reported to indicate the erythrocyte mass in amphibians (Allender and Fry, 2008).

Erythrocyte count is a simple blood parameter measurement performed to determine the number of erythrocytes in a unit volume of blood that has been diluted in an isotonic solution (Salakij, 2005). The count is important because erythrocytes contain hemoglobin, which carries oxygen to the tissues. The number of erythrocytes reflects how much oxygen the blood can carry to the tissues (Hutchinson and Szarski, 1965; Rouf, 1969; Sinha, 1983). The erythrocyte count can help diagnose different kinds of anemia and other conditions affecting erythrocytes (Bain et al., 2012; Salakij, 2005). The erythrocytes are counted using counting chamber, also called hemocytometer, or automatic counter such as a flow cytometer. The results are expressed as the number of erythrocyte in 1 μL of blood (Salakij, 2005).

Leukocyte count is a measurement that determine the number of leukocytes in a unit volume of blood. When the body has an infection or allergic reaction, the leukocyte number may be raised in response to the symptom. The percentage of each type of leukocyte in a species can change from the normal level if there is disturbance in the body. An increased number of leukocytes can be due to a blood cancer, such as leukemia or lymphoma (Salakij, 2005).

Plasma proteins are a group of proteins in circulating blood including prealbumin, albumin (most abundant of blood plasma protein), globulin and fibrinogen. The measurement of plasma total proteins can help diagnose different kinds of hypoalbuminemia, hypoglobulinemia, hyperglobulinemia and hyperfibrinogenemia (Salakij, 2005).

The values obtained from the analysis of the hematological parameters are normally used in comparison with the normal reference values in a specific species to assess the overall health of the animals. Hematological normal reference value is a set

of value resulting from blood sample test of healthy animals, that used by a health professional to interpret a set of medical test results. It is a basis for a physician to interpret a set of test results for a particular domestic animal and a human patient. The hematological test result that falls out of the range of normal reference values can indicate abnormality of the patient's health status and it is needed for further advanced investigation. The normal reference range interval, which normally comprises a range of mean ± 2SD (Figure 2-1), indicates the limits that should cover 95% of normal subjects (Bain et al., 2012; Salakij, 2005).

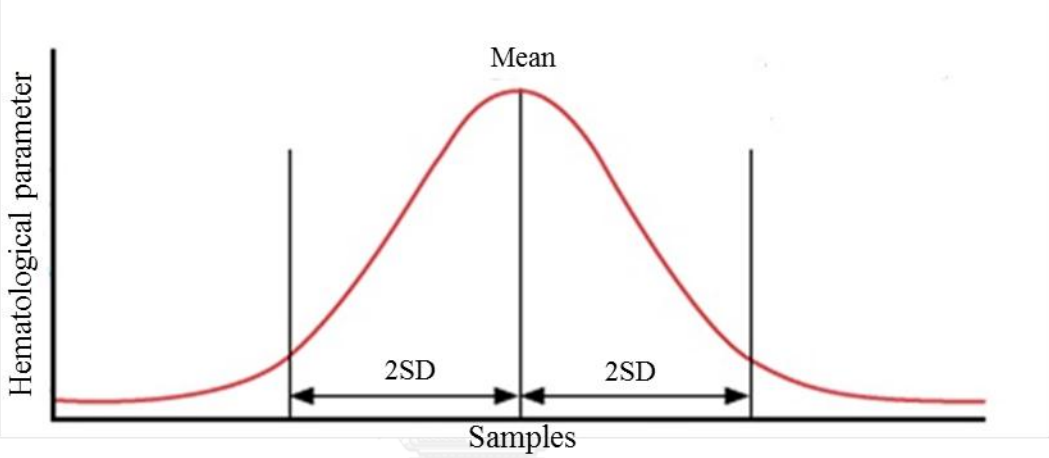

Figure 2-1 Example of establishing a normal reference range of a hematological parameter

2. Hematology of amphibian

Data on hematological parameters of human and domestic mammals are well documented, but for amphibian, it is still limited. Some papers have reported the characteristics of amphibian blood (Arikan and Cicek, 2014; Arıkan and Cicek, 2010; Arserim and Mermer, 2008; Bricker et al., 2012; Canfield, 1998; Claver and Quaglia, 2009; Das and Mahapatra, 2012; Kuramoto, 1981; Liu et al., 2013; Orr et al., 1986; Thomas and Maclean, 1975). Using hematological parameters as indicator of effects from environmental changes has been reported in some *Rana* species (Arserim and Mermer, 2008; Palenske and Saunders, 2003; Sinha, 1983; Weathers, 1975; Zhelev et al., 2006). Using of hematological parameter values as an indicator of the health status were reported in tree frog and American bullfrog (*Hyla septentrionalis* and *Rana catesbeiana*; Carmena-Suero et al., 1980), common toad (*Bufo arenarum*; Cabagna et

al., 2005), European toad (*Bufo bufo*; Donmez et al., 2009), lycian salamander (*Lyciasalamandra fazilae*; Tok et al., 2009), hellbender (*Cryptobranchus alleganiensis alleganiensis*; Burgmeier et al., 2011), common frog (*Rana temporaria*; Omonona and Ekpenko, 2011) and Indian rhacophorid tree frog (*Polypedates maculatus*; Mahapatra et al., 2012). Using hematological parameter values as the stress indices in breeding season was also reported in mole salamanders (*Ambystoma talpoideum*; Davis and Maerz, 2008). Additionally, hematological normal reference values have been reported in some amphibian species including African clawed frog (*Xenopus laevis*; Chang et al., 2015; Wilson et al., 2011), Australian green tree frog (*Litoria caerulea*) and white-lipped tree frog (*Litoria infrafrenata*; Young et al., 2012).

3. Rice field frog

The rice field frog, *Hoplobatrachus rugulosus* (Wiegmann, 1834) is a well-known species of amphibian in the order Anura, family Dicroglossidae. This frog is also known as Chinese edible frog, East Asian bullfrog and Taiwanese frog. The status of *H. rugulosus* is identified as the least concern according to the assessment information of the IUCN Red List Category and Criteria (Diesmos et al., 2004). It has a wide distribution, tolerance of a broad range of habitats, and its population is presumably large and appears to be stable at present.

The rice field frog is a medium size amphibian which has the total length of approximately 90-180 mm. The external morphology is described in Taylor (1962) and Uk-katawewat (1997) as follows. The body is long with arms and legs moderately short. Interorbital space is much narrower than upper eyelid. Snout is oval and nostril much nearer eye than median tip of snout. Jaw is forming slight shelf below eye. Tympanum is large and covered with skin. Distinction of tympanum is about one third of the eye diameter. Fingers are obtusely pointed and the first finger is longer than second one. Subarticular tubercles are large and flat. Tibiotarsal articulation reaches front of eye. Toes have nearly fully webbed on hind legs. The skin have extremely granular and rough with many scattered, small to large, many interrupted predominant horny ridges of skin running down the back and upper surface. Colors of body are

brown to greenish gray above with scattered dark spots on the back and legs. Ventrum is white. Special character of male frog is the throat, which is mottled with brown. The weight are 200-400 g when fully grown. The female frogs are larger than males (Figure 2-2).

Figure 2-2 The rice field frog *H. rugulosus* (Wiegmann, 1834)

The populations of *H. rugulosus* distribute in wetlands and paddy fields throughout central, southern and south-western China including Taiwan, Hong Kong and Macau to Myanmar through Thailand, Lao People's Democratic Republic, Vietnam and Southern Cambodia to the Thai-Malay peninsula (Diesmos et al., 2004). They are commonly found in many agricultural areas in Thailand (Amatyakul, 1995). However, their natural habitats in Thailand are disturbed and very limited, natural populations can still be found in Nan Province, northern Thailand, and lowland sites in other provinces from central to southern Thailand (Figure 2-3; Pansook et al., 2012). This frog is an economically important animal of Thailand. It also contributes to economics of many other countries because it is food of the local people (Amatyakul, 1995).

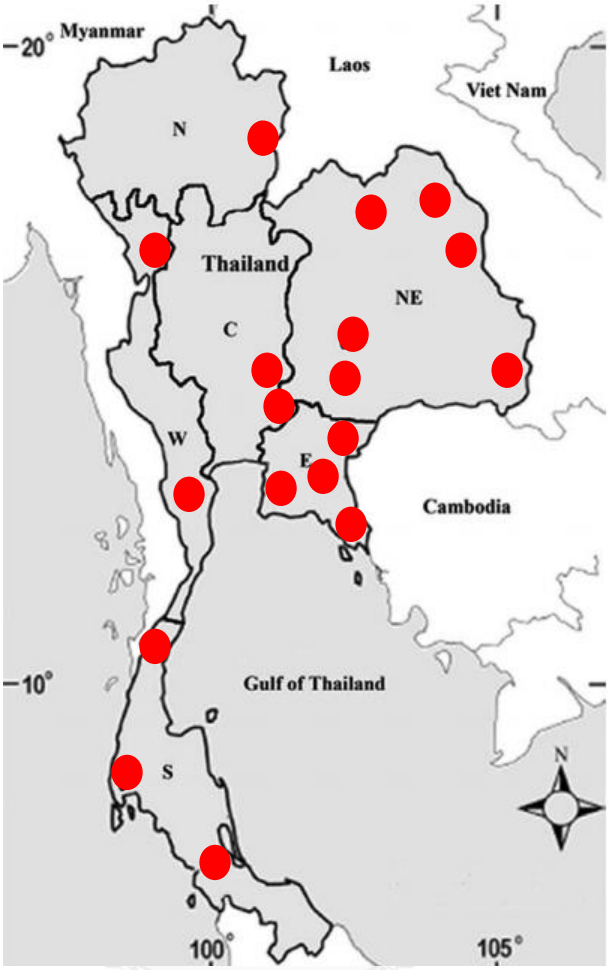

Figure 2-3 Geographic distribution of *H. rugulosus* in Thailand, the highlight red color areas showed distribution of the frog (modified from Pansook et al., 2012)

4. Frog farming in Thailand

In Thailand, frog farming is increasing and becomes more popular in response to increasing market demand. *H. rugulosus* is the most popular frog species being cultured in farm system in Thailand. It is also been exported to China, Hong Kong, Singapore, Taiwan, the European Union and the United States (Amatyakul, 1995). Like other farmed animals, farm frogs often have stress and diseases due to limitation of space. The most common disease of frogs, red-leg disease, is a result of bacterial infection (*Aeromonas hydrophila*, gram-negative bacteria). The name of disease comes from the fact that the bacteria cause the blood vessels in a frog's legs to congest, swell and turn red. This condition can be fatal. Head tilt disease is also

commonly found in farmed frogs. The frog with this disease cannot move correctly. This disease associates with *Flavobacterium* (gram-negative bacteria) infection and the neurologic impairment (Densmore and Green, 2007). Currently, the health status of farmed frogs is generally based on visual inspection because there is no report about normal reference value of hematological parameters of this frog that can be used to assess the overall health status of the frogs and monitor the spread of disease in the ponds.

In Thailand, frog farming is widely operated as a commercial farming (Thaksin, 2008). Many frog farms were found in the central part of Thailand such as Phra Nakhon Sri Ayutthaya and Nakhon Nayok Provinces and northern part of Thailand such as Mae Hong Son, Nan and Chiang Mai Provinces. In case of the northern part of Thailand, many farmer communities have been encouraged by the government to raise the frogs in their farms for their additional income. Since, many frog farms aim to the incremental consequence, so the number of frogs per pond area is not suitable for culture. This might be a cause of frog disease in the pond.

The Huai Hong Khrai Royal Development Study Center was founded for research and experimentation to develop methods that will be suited to the development needs of people of Thailand in hilly northern region. This center locates in Khun Mae Kuang National Forest Reserve, Doi Saket District, Chiang Mai Province. It was established under the initiative of His Majesty King Bhumibol Adulyadej in 1982. The center has conducted researches on the conservation of watersheds, reforestation, forest conservation and agricultural development such as development of crop cultivation (integrated farming system involving fruit trees, vegetable crops and livestock in the same plot of land), development of milk cows and livestock, development of fishery (training and management of fishery, harvesting techniques), and conservation and development of frog farming (dissemination of technical know-how and example of frog farming). The center also provides people who live in the surrounding villages with services in many fields, such as supporting animal husbandry activities and plant seeds and also giving advice and guidance on techniques which farmers and other people interested can apply to their daily occupations (Huai Hong Khrai Royal Development Study Center., 2005). The

conservation and development of frog farming at the Huai Hong Khrai Royal Development Study Center aims to function as a complete one-stop service center. The center has been given advice and guidance on techniques in frog farming for the local people. The center has nurtured standardized cultivation system operated under supervision of official expertise in order to provide an example of standardized frog farm system for local people (Figure 2-4). So, this center was an appropriate source of farm raised *H. rugulosus* samples for the hematological study of the frog in farm system.

Figure 2-4 Frog farm in Huai Hong Khrai Royal Development Study Center, Chiang Mai Province, northern part of Thailand

CHAPTER III
HEMATOLOGY OF WILD CAUGHT *Hoplobatrachus rugulosus* IN NORTHERN THAILAND

Introduction

Monitoring the health of wildlife is very important for their conservation and management. Among general health assessment approaches, hematological analyses are viewed as reliable methods to determine the health status in mammals and other vertebrates. Changes in some hematological parameters compared with the reference values may be used as evidences of physiological disturbances such as xenobiotic exposure, stress and diseases in vertebrates (Bloom and Brandt, 2008; Davis et al., 2008). Among the hematological parameters of peripheral blood that have been frequently used to assess the health status of animals are the packed cell volume (PCV) or hematocrit, erythrocyte count, leukocyte count and differential leukocyte count.

Data on the hematological parameters of human and domestic mammals are well documented, but remain limited for amphibians and reptiles. With respect to amphibians, the use of hematological parameters for health assessment has been reported in some species such as the common toad (*Bufo arenarum*) in agricultural areas in Argentina (Cabagna et al., 2005), the marsh frog (*Rana ridibunda*) in an industrial area in Bulgaria (Zhelev et al., 2006), the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in a pesticide contaminated river in southern Indiana, USA (Burgmeier et al., 2011) and the northern leopard frog (*Lithobates pipiens*) in pesticide contaminated areas in Canada (Shutler and Marcogliese, 2011). However, reference data on the hematological parameters of wild populations of frog species is scarce compared with the diversity of animals in this taxonomic group, even though it is crucial for their health assessment.

The rice field frog, *Hoplobatrachus rugulosus* (Wiegmann, 1834) is an anuran amphibian in the family Dicroglossidae with a widespread distribution from central China and Myanmar to Thailand and peninsular Malaysia, where they are commonly found in wetlands and paddy fields (Diesmos et al., 2004). Even though their natural

habitats in Thailand have been disturbed and are very limited, natural populations can still be found in Nan Province, northern Thailand, and lowland sites in other provinces from central to southern Thailand (Pansook et al., 2012). This frog is economically important in Thailand because it is used as food by the local people. It also has the potential to be used as a model for research in many fields such as physiology, reproductive biology and ecotoxicology.

At present, wild populations of *H. rugulosus* are of concern due to human-mediated over harvesting, habitat disturbances, stress and diseases, but the health status of these natural populations is unknown. Until now, the hematological parameters in the natural populations of *H. rugulosus* are still undocumented. Only the hematological aspects of a closely related species, the common Indian frog (*Rana tigrina*) have been reported (Singh, 1977a; Singh, 1977b; Singh, 1978). Therefore, the purpose of this study was to identify the morphological characteristics of different peripheral blood cells and to determine the hematological parameters of the peripheral blood in *H. rugulosus*. Data obtained from this study would then form the fundamental basis for monitoring of the health status of this natural anuran population.

Materials and Methods

1. Frog collection

1.1 Frog collection site

Adult *H. rugulosus* were collected from organic rice fields (UTM 0686779 2047187, zone line 47Q) with no history of herbicide usage for more than 10 years (Jantawongsri et al., 2015; Thammachoti et al., 2012) in Lai-nan Sub District, Wiang Sa District, Nan Province, northern part of Thailand.

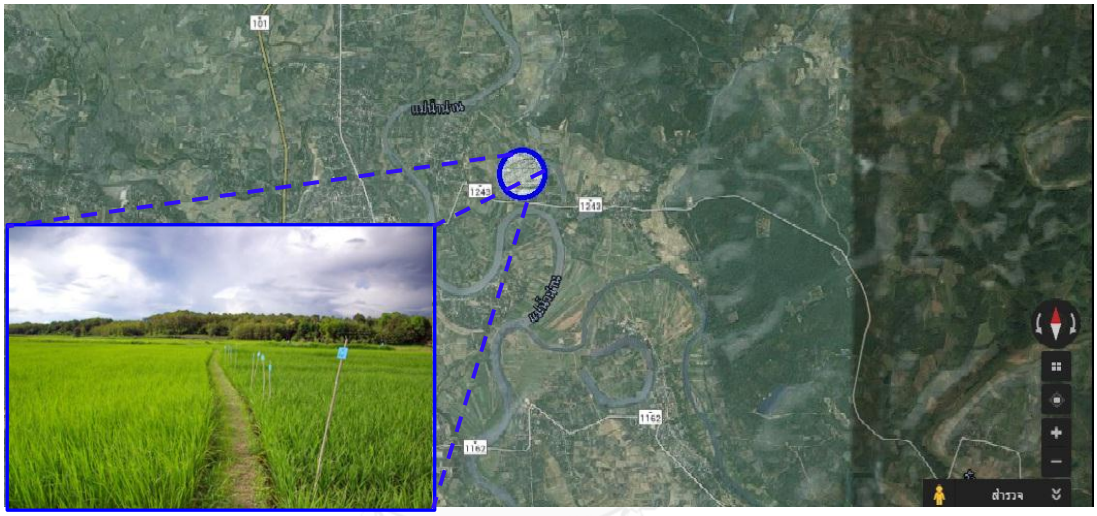

Figure 3-1 The frog collection site in Lai-nan Sub District, Wiang Sa District, Nan Province, Thailand **(**modified from https://www.google.co.th/maps/Wiang+Sa+District+Nan/@18.5477561,100.7531231,13.5z)

Nan Province has a total area of 11,472 km^2 with human population of more than 475,000 individuals. Most people who live in the province are farmers, especially people who live in the lowland at central and southern part of the province where Nan River runs through the areas (Wiang Sa District Agricultural Extension Office , 2012). Most of agricultural activities in these areas are generally rice cultivation and vegetable farming (Thadaniti and Prachuabmoh, 2005). Previous report by Wiang Sa District Agricultural Extension Office (2012) showed that more than 97 % of the land is used for agricultural purposes (Figure 3-1). As a result, Nan Province still has agricultural areas which are wetland habitats of the rice field frogs.

1.2 Sampling season

Adult *H. rugulosus* were collected during the rainy season (July to August 2014). The average temperature in the sampling period was 27.7 °C. The rainy season in this study was defined based on a climograph plotted between mean temperature and total rainfall (Walter et al., 1975). The climograph indicated that the rainy season ranged from July to September in 2014 (Figure 3-2).

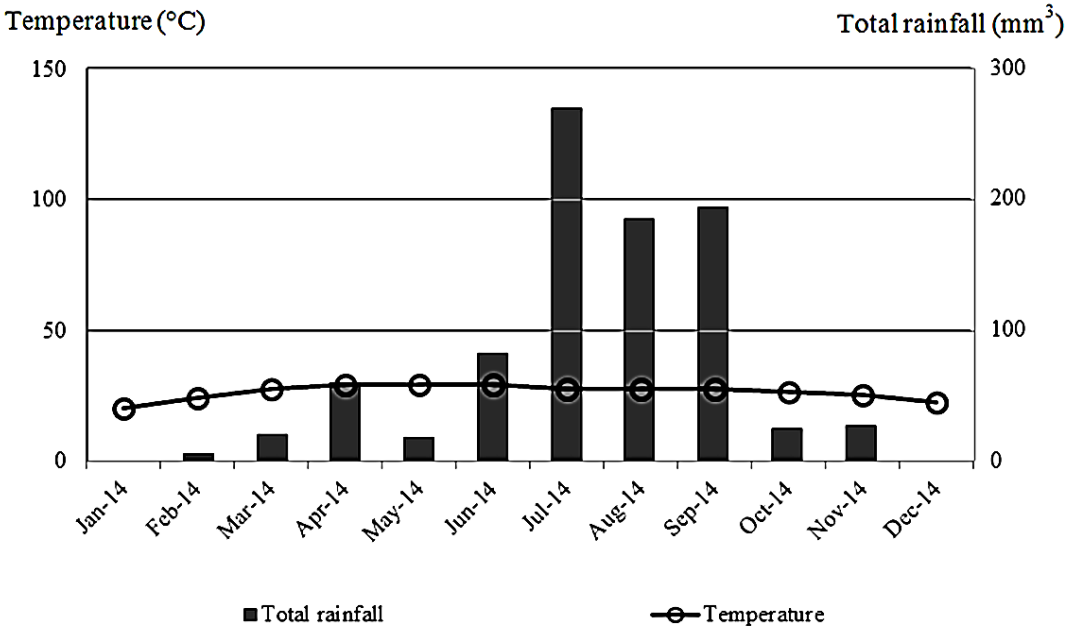

Figure 3-2 Climograph of the frog collection site in Lai-nan Sub District, Wiang Sa District, Nan Province in 2014

1.3 Frog survey and collection

The collection of *H. rugulosus* was performed 2 times in July and October in 2014. Thirty-three adult frogs (male = 17, female = 16) were collected by the visual encounter survey method (Crump and Scott Jr, 1994) from its natural habitat. After transportation to a laboratory at the Chulalongkorn University Forest and Research Station at Nan Province, the frogs were acclimatized for 1 day in 7 L plastic aquaria (1 frog per aquarium). During the acclimatization, the frogs were fed with mealworm (*Tenebrio molitor*).

2. Morphometry and blood sampling

After acclimatization, the frogs were anesthetized in ice slurry. Snout-vent length (SVL) and body weight (BW) of each frog were recorded. Blood samples (0.5 mL per 100 g body weight) were collected from each frog by cardiac puncture (Heatley and Johnson, 2009) using 25-gauge needle with heparinized tuberculin syringe and transferred to microcentrifuge tube. The samples were stored in ice bucket during an hour of PCV, hemocytometer counting and blood smear processes.

3. Hematology

3.1 Blood smear and staining

The blood sample of the frog was smeared on glass slides immediately. The air-dried blood film was fixed in absolute methanol for 2 minutes. Five blood smear slides were prepared for 1 individual. The slides were stained for 20 minutes in Giemsa solution (Appendix B-I) at pH 7.0 (Bain et al., 2012). Then the stained slides were rinsed in phosphate buffer saline and washed vigorously in tap water to remove the excess dye deposits. After that, the slides were transfered to n-butanal for 3 minutes, cleared in xylene for 5 minutes and mounted in Permount mountant. The blood smear slides were observed for blood cell morphology and determination of the hematological parameters using light microscope at 400× magnification.

3.2 Hematological parameters

3.2.1 Morphology and morphometry of blood cells

Immature and mature erythrocytes were counted in 10 fields with an aid of grid ocular micrometer and the percentage of immature erythrocyte was calculated (Briggs and Bain, 2012). For morphometric study of blood cell size, blood smears from 10 frogs (5 frogs per sex) were randomly selected out of 33 frogs. On each blood smear slide of a frog, the length and width of 30 randomly selected mature erythrocytes and their nuclei, and 30 randomly selected thrombocytes were measured using the Image-Pro Plus 6.00 software (Media Cybermetics, Silver Spring, USA) from their digital images taken with a digital camera (Cannon EOS 550D). Selection of the areas from good spread blood film with no overlap of the cells and the cell number (30 cells) used in these measurements were based on a previous study by Kuramoto (1981). Likewise, the diameters of 30 randomly selected cells for each of lymphocytes, monocytes, neutrophils, basophils, eosinophils and immature erythrocytes, were also measured in the same manner. The mean value of each morphometric parameter in each frog was calculated from 30 cells, and the grand mean from 5 frogs was finally calculated. The erythrocyte cell and nuclear areas (EA and NA, respectively) were calculated according to the formula of the area of an elliptical shape (length × width × $\pi/4$), and the nucleocytoplasmic ratio (NA/EA) was calculated (Arıkan and Cicek, 2010). In the same way the immature erythrocyte cell and nuclear areas (IEA and NIEA, respectively) were calculated according to the formula of the area of a circular shape [$\pi \times (\text{diameter}/2)^2$], and used to derive the nucleocytoplasmic ratio (NIEA/IEA).

3.2.2 Erythrocyte and leukocyte counts

In this study, erythrocyte and leukocyte counts were determined manually using a hemocytometer (Tharp and Woodman, 2002). The blood sample from each frog was primarily diluted with small amount of Natt and Herrick's solution (Natt and Herrick, 1951) in diluting pipette. Then, the Natt and Herrick's solution (Appendix B-II) was taken continuously with the same pipette that already contained blood sample until the mixed solution reaching the scale marked 101 (for erythrocyte

count) or 11 (for leukocyte count). The pipette was rotated gently until the blood sample and the solution was well mixed. The solution inside was 1:200 dilution of blood sample in the Natt and Herrick's solution. Then, a cover slip was placed in the position that covered the Neubauer ruling area of the counting chamber. The pressure was slightly applied on a rubber tube that connected with the pipette so that a drop of blood solution was in hanging position. The tip of the pipette (with a hanging drop) was positioned to touch against the edge of the cover slip on the counting chamber. Then, the chamber was getting filled with the blood solution. During this process, air bubble should not present inside the chamber and the blood solution should not over filled on the ruling area. The counting chamber was left without disturbance about 3 minutes. After that, the chamber was placed on the stage of a light microscope. Blood counting was performed using light microscope at 400× magnification. The total number of viable cells/µL was obtained by the following equation.

$$\text{Total number of viable erythrocyte cells/µL} = \text{average viable erythrocyte cell count} \times 5{,}000$$

$$\text{Total number of viable leukocyte cells/µL} = \text{average viable leukocyte cell count} \times 1{,}000$$

3.2.3 Packed cell volume

The blood sample of each frog was of each frog transferred to a microcapillary tube. The blood-filled microcapillary tube was sealed at one end with Critoseal wax before placing in a microhematocrit centrifuge (Centurion Scientific LTD, *1020 series*). Then, the samples were centrifuged at 8,700 ×g for 10 minutes (Salakij, 2005). After centrifugation, the microcapillary tubes were placed in a microhematocrit reader to evaluate the hematocrit value according to the manufacturer’s instruction on the microhematocrit reading device.

3.2.4 Differential leukocyte count

The Giemsa stained blood smear slides were examined using 400× magnification under a light microscope in the areas that the cell morphology was normal and the cells were well-dispersed. A total of 200 cells were counted in a strip

running manner adapted from a straight-edge method (Salakij, 2005) for each leukocyte type including lymphocyte, monocyte, neutrophil, basophil and eosinophil. Then, the percentage of each cell type was calculated.

3.2.5 Plasma total protein determination

The blood sample of each frog (0.5 - 0.7mL) of each frog had been transferred to a microcentrifuge tube and centrifuged at 1,500 ×g at 4°C for 15 minutes. The plasma separated by centrifugation was collected and transferred into a new microcentrifuge tube and stored at -20°C. The plasma samples were subjected to the procedure of total protein determination by Bradford assay (Bradford, 1976). The samples were analyzed in a 96-well plate using a stock solution of 25 µg/mL bovine serum albumin (BSA) as a standard protein. The BSA standard and the plasma samples were diluted with phosphate buffered saline (PBS; Appendix B-I). For BSA standard, serial dilution was performed to obtain the concentration from 0.78125 to 25 µg/mL. The plasma samples were diluted with PBS at 1:1,000 and 1:10,000 dilutions. One hundred microliters of standards, quality control sample (QC; BSA 10 µg/mL), blank (PBS) and samples were added in duplicate into each well. A 100 µL aliquot of Bradford solution (Sigma) was added into each well using a muti-channel pipette. The microplate filled with the samples was shaken with PSU 2-T mini shaker for a few minutes at room temperature before measuring for absorbance by Multiskan EX microplate reader at 595 nm (Redinbaugh and Campbell, 1985). The average absorbance of each standard was corrected by subtracting with an average absorbance of PBS blank. A standard calibration curve was constructed by plotting the corrected absorbance on a vertical (Y) axis versus the corresponding BSA concentration on horizontal (X) axis followed by linear regression analysis using Microsoft Excel software (Microsoft Corporation, Redmond, USA). The plasma protein concentration of the samples were determined using linear regression equation of the standard curve.

4. Statistical Analyses

All data of each blood parameter were tested for normal distribution by the Kolmogorov-Smirnov test and homogeneity of variance. The hematological

parameters were compared between sexes by Student *t*-test for the data that are normally distributed or by Mann-Whitney rank sum test for the data that are not normally distributed (Zar, 1998). Sigma Plot version 11.0 software (Systat Software, San Jose, USA) was used for the data analysis.

Results

1. Morphology and morphometry of blood cells

Peripheral blood cells of *H. rugulosus* were classified into erythrocytes, immature erythrocytes, thrombocytes and leukocytes under light microscope. Mature erythrocytes were large elliptically shaped cells with a centrally located elliptical shaped nucleus with dense basophilic chromatin. The nuclear membrane was very prominent under light microscopy and the cytoplasm stained light blue to colorless (Figure 3-3A). The mean values of nuclear length and width were 6.95 ± 1.18 μm and 4.69 ± 1.15 μm in males, respectively and 6.80 ± 1.08 μm and 4.55 ± 0.62 μm in females, respectively. The mean values of cell length and width were 17.73 ± 1.45 μm and 11.17 ± 0.99 μm in males, respectively and 18.18 ± 1.39 μm and 11.83 ± 1.08 μm in females, respectively (Table 3-1).

In this study, immature (polychromatic) erythrocytes were found in the circulating blood of this frog species. They were spherical with a centrally located round nucleus that was larger and less basophilic staining than that of mature erythrocytes, while the cytoplasm stained light blue or colorless (Figure 3-3A). The mean values of cell diameter were 14.80 ± 2.36 μm in males and 15.03 ± 2.04 μm in females. The mean values of nuclear diameter were 7.29 ± 1.26 μm in males and 7.33 ± 1.27 μm in females (Table 3-1). Comparison of the nucleocytoplasmic ratios (Table 3-1) showed that the immature erythrocytes had a significantly higher (1.69-fold) nucleocytoplasmic ratio than that of the mature erythrocytes. In this study, the erythrocytes with mitotic figure were also evidented (Figure 3-3H).

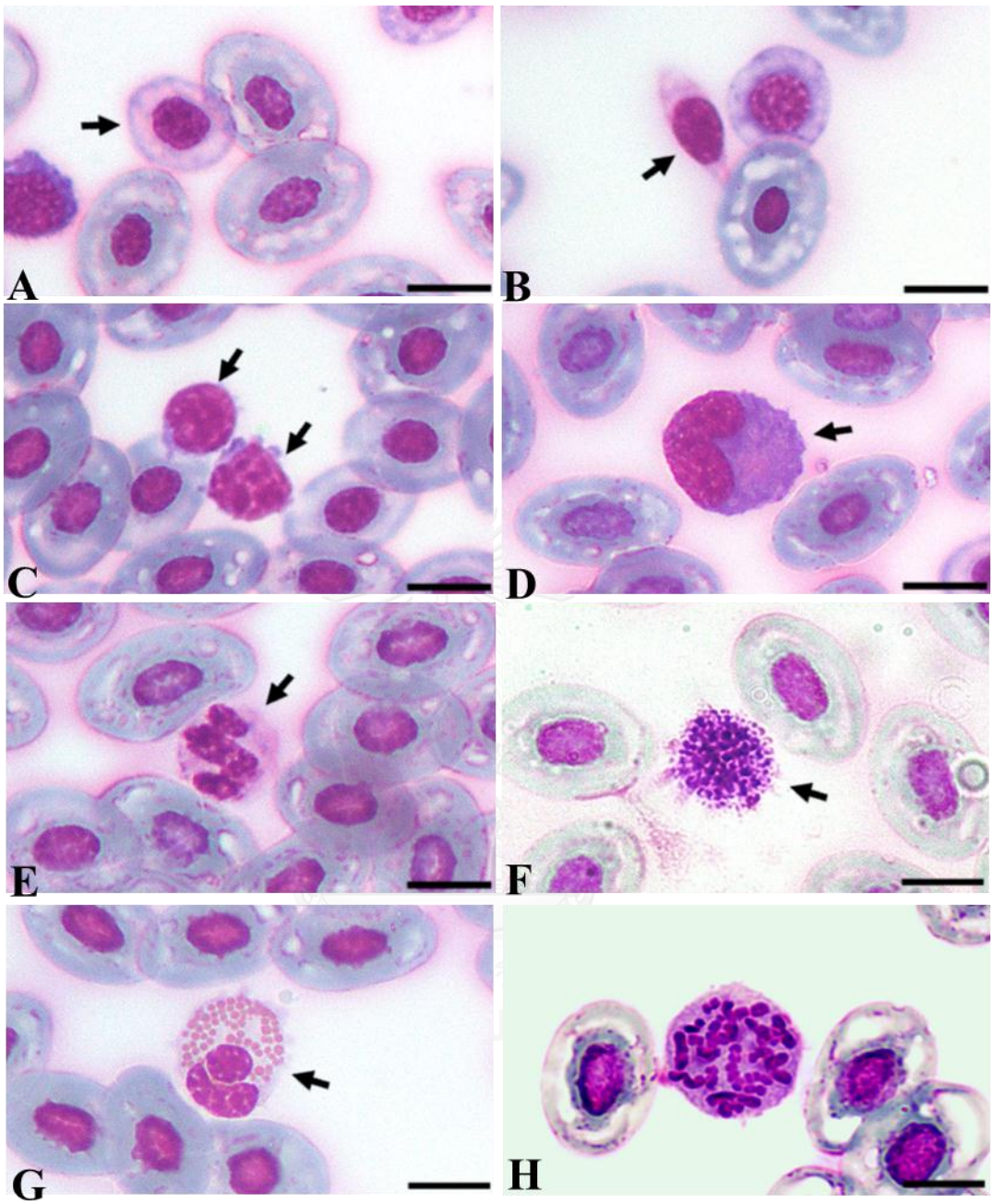

Figure 3-3 Light micrographs showing the different types of blood cells in *H. rugulosus.* Erythrocytes were classified as (**A**) mature and immature (arrow) erythrocytes. (**B**) Thrombocytes (arrow). Leukocytes were classified as (**C**) lymphocytes (arrows), (**D**) monocytes (arrow), (**E**) neutrophils (arrow), (**F**) basophils (arrow), (**G**) eosinophils (arrow), (**H**) erythrocyte with mitotic figure (arrow). Bar = 10 µm; Giemsa stain.

Table 3-1 Morphometric data of mature and immature erythrocytes (mean ± SD) of *H. rugulosus* collected from Nan Province from July to August 2014

Characters	Sex[a]	Mean	SD	Range
Erythrocyte length (μm)	Male	17.73	1.45	14.64-23.22
	Female	18.18	1.39	15.31-22.47
	Both sexes	17.96	1.44	14.64-23.22
Erythrocyte width (μm)	Male	11.17	0.99	8.72-14.40
	Female	11.83	1.08	9.25-15.89
	Both sexes	11.50	1.09	8.72-15.89
Erythrocyte area (EA; μm^2)	Male	155.32	16.80	117.07-213.79
	Female	169.41	24.49	117.78-267.11
	Both sexes	162.36	22.12	117.07-267.11
Erythrocyte nuclear length (μm)	Male	6.95	1.18	4.84-10.51
	Female	6.80	1.08	4.20-9.78
	Both sexes	6.87	1.14	4.20-10.51
Erythrocyte nuclear width (μm)	Male	4.69	1.15	2.34-8.36
	Female	4.55	0.62	3.06-6.48
	Both sexes	4.62	0.93	2.34-8.36
Erythrocyte nuclear area (NA; μm^2)	Male	26.13	10.40	12.15-64.95
	Female	24.43	5.51	10.29-38.99
	Both sexes	25.28	8.35	10.29-64.95
Erythrocyte nucleocytoplasmic ratio (NA/EA)	Male	0.17	0.07	0.08-0.47
	Female	0.15	0.04	0.06-0.26
	Both sexes	0.16	0.06	0.06-0.47
Immature erythrocyte diameter (μm)	Male	14.80	2.36	9.64-20.28
	Female	15.03	2.04	9.64-20.39
	Both sexes	14.91	2.20	9.64-20.39
Immature erythrocyte area (IEA; μm^2)	Male	176.28	54.07	72.94-322.91
	Female	180.69	48.15	72.94-326.58
	Both sexes	178.49	51.16	72.94-326.58
Immature erythrocyte nuclear diameter (μm)	Male	7.29	1.26	4.50-9.97
	Female	7.33	1.27	4.50-9.97
	Both sexes	7.31	1.26	4.50-9.97
Immature erythrocyte nuclear area (NIEA; μm^2)	Male	43.03	14.71	15.89-78.03
	Female	43.45	14.91	15.89-78.03
	Both sexes	43.24	14.78	15.89-78.03
Immature erythrocyte nucleocytoplasmic ratio * (NIEA/IEA)	Male	0.27	0.14	0.07-1.01
	Female	0.26	0.13	0.06-1.04
	Both sexes	0.27	0.14	0.06-1.04

Remarks: [a] Data are derived from 5 male, 5 female or both (10 male and female) frogs.

[*] Significant difference in the mean nucleocytoplasmic ratios between mature erythrocytes (NA/EA) and immature erythrocytes (NIEA/IEA), Student's *t*-test ($P \leq 0.05$)

Thrombocytes were round to elliptical-shaped cells of a smaller size (1.29-fold and 1.93-fold in length and width, respectively) than mature erythrocytes and contained an elliptical nucleus. The clear cytoplasm was scarce and stained light blue or colorless (Figure 3-3B). The mean values of cell length and width were 13.02 ± 3.54 μm and 6.57 ± 1.25 μm in males, respectively and 14.85 ± 2.36 μm and 7.54 ± 1.18 μm in females, respectively (Table 3-2). The cells were sometimes found grouped together on the blood smears.

Table 3-2 Morphometric data of thrombocytes (mean ± SD) of *H. rugulosus* collected from Nan Province from July to August 2014

Characters	Sex[a]	Mean	SD	Range
Thrombocyte length (μm)	Male	13.02	3.54	6.30-20.14
	Female	14.85	2.36	7.68-19.55
	Both sexes	13.93	3.14	6.30-20.14
Thrombocyte width (μm)	Male	6.57	1.25	4.20-9.92
	Female	7.54	1.18	4.50-10.74
	Both sexes	7.05	1.31	4.20-10.74

Remark: [a] Data are derived from 5 male, 5 female or both (10 male and female) frogs.

The leukocytes were separated into agranulocytes and granulocytes and then further classified into two and three cell types, respectively, based on the morphological characteristics of the Giemsa stained blood smears. Agranulocytes were classified into lymphocytes and monocytes. Lymphocytes were round or slightly elliptical shaped cells that contained small amount of cytoplasm. The nucleus was compact and dark stained, positioned centrally in the cell and covered by a light-blue stained cytoplasm (Figure 3-3C). The mean values of cell diameter were 11.02 ± 2.87 μm in males and 11.00 ± 2.51 μm in females (Table 3-3). Monocytes had a relatively higher cytoplasmic to nuclear ratio than large lymphocytes and contained a round, kidney- or horseshoe-shaped nucleus adjacent to the cell edge. The nucleus had less intensely stained chromatin than that of the lymphocytes (Figure 3-3D). The mean values of cell diameter were 12.46 ± 2.45 μm in males and 11.63 ± 2.28 μm in females (Table 3-3). Monocytes may be found varrying in size in the blood smear.

Granulocytes were classified into three cell types (neutrophils, eosinophils and basophils) based on the characteristics of their nuclei and cytoplasmic granules. Neutrophils were round cells, marginally larger (1.04-fold) than monocytes and had a multiple-lobed nucleus, like in humans. However, some cells contained nuclei that looked U-shaped. The cytoplasm contained fine granules and stained light purple (Figure 3-3E). The mean values of cell diameter were 12.84 ± 2.08 μm in males and 12.23 ± 2.07 μm in females (Table 3-3). Basophils were fairly large cells, slightly (1.03-fold) larger than large lymphocytes, and were characterized by the presence of round highly basophilic (dark blue) granules of various sizes in the cytoplasm. The inconspicuous round nucleus was positioned in the center of the cell (Figure 3-3F). The mean values of cell diameter were 13.42 ± 2.11 μm in males and 13.73 ± 2.26 μm in females (Table 3-3). Eosinophils were sized in between neutrophils and monocytes with a less segmented nucleus, and relatively large cytoplasmic granules of a round to elliptical shape that stained red brown (Figure 3-3G). The mean values of cell diameter were 12.95 ± 3.30 μm in males and 11.75 ± 2.42 μm in females (Table 3-3).

Table 3-3 Morphometric data of leukocytes (cell diameter; mean ± SD) of *H. rugulosus* collected from Nan Province from July to August 2014

Diameters	Sex[a]	Mean	SD	Range
Lymphocytes (μm)	Male	11.02	2.87	5.94-18.96
	Female	11.00	2.51	5.18-18.74
	Both sexes	11.01	2.69	5.18-18.96
Monocytes (μm)	Male	12.46	2.45	6.66-18.31
	Female	11.63	2.28	8.03-19.73
	Both sexes	12.04	2.40	6.66-19.73
Neutrophils (μm)	Male	12.84	2.08	9.52-18.11
	Female	12.23	2.07	7.83-15.79
	Both sexes	12.58	2.08	7.83-18.11
Basophils (μm)	Male	13.42	2.11	10.06-17.49
	Female	13.73	2.26	10.08-17.49
	Both sexes	13.60	2.17	10.06-17.49
Eosinophils (μm)	Male	12.92	3.30	6.90-20.15
	Female	11.75	2.42	7.33-18.48
	Both sexes	12.33	2.95	6.90-20.15

Remark: [a] Data are derived from 5 male, 5 female or both (10 male and female) frogs.

2. Hematological parameters

The hematological parameters of the peripheral blood of wild caught *H. rugulosus* are summarized in Table 3-4 and 3-5. The mean erythrocyte count (derived from hemocytometer counting) of male frogs was numerically slightly (1.08-fold) higher but not significantly different from that of female frogs (t = 0.38, df = 31, P = 0.71). Likewise, the leukocyte count was numerically (1.25-fold) but not significantly higher in male frogs than in female frogs (Mann-Whitney U, T = 261.5, P = 0.71). However, a significant difference in the mean PCV between the sexes was found, being 1.22-fold higher in male frogs than in female frogs (t = 2.84, df = 31, P = 0.01). Mean plasma total protein of male frogs (31.16 ± 14.05 mg/mL) was not significantly different from that of the female frogs (30.62 ± 13.85 mg/mL, t = 0.11, df = 31, P = 0.91).

Hematological examination of the peripheral blood smear slides revealed sex-related differences in some leukocyte parameters (Table 3-5), where the mean values of differential lymphocyte count of male frogs is significantly higher than that of the

female frogs (1.1-fold, t = 2.74, df = 31, P = 0.01). Mean values of differential neutrophil count was also significantly higher in male frogs than in female frogs (2.0-fold, t = 2.12, df = 31, P = 0.04).

Table 3-4 Blood cell counts, packed cell volume (PCV) and plasma total protein (mean ± SD) of *H. rugulosus* collected from Nan Province from July to August 2014

Parameters	**Sex**[a]	**Mean**	**SD**	**Range**
Erythrocyte ($\times 10^6$ cells/μL)	Male	0.64	3.98	1.85-16.65
	Female	0.60	2.81	1.80-10.40
	Both sexes	0.62	3.42	1.80-16.65
Leukocyte ($\times 10^3$ cells/μL)	Male	5.31	3.74	1.13-13.00
	Female	4.26	1.74	1.68-6.75
	Both sexes	4.80	2.95	1.13-13.00
Immature erythrocyte * (%)	Male	5.46	1.59	3.00-8.83
	Female	8.19	2.15	4.16-12.50
	Both sexes	6.78	2.31	3.00-12.50
PCV * (%)	Male	30.70	6.07	20.50-39.00
	Female	25.09	4.85	11.00-32.00
	Both sexes	27.99	6.13	11.00-39.00
Plasma total protein (mg/mL)	Male	31.16	14.05	15.60-52.56
	Female	30.62	13.85	7.83-64.18
	Both sexes	30.90	13.73	7.83-64.18

Remarks: [a] Data are derived from 17 male, 16 female or both (33 male and female) frogs.

[*] Significant difference of mean parameter values between males and females, Student's *t*-test ($P \leq 0.05$)

Table 3-5 Differential leukocyte counts (mean ± SD) of *H. rugulosus* collected from Nan Province from July to August 2014

Parameters	**Sex**[a]	**Mean**	**SD**	**Range**
Differential lymphocyte *	Male	57.49	6.56	45.00-69.00
(%)	Female	51.66	5.57	42.50-62.50
	Both sexes	54.66	6.69	42.50-69.00
Differential monocyte	Male	30.88	7.69	20.00-46.00
(%)	Female	36.09	7.75	21.50-49.50
	Both sexes	33.41	8.05	20.00-49.50
Differential neutrophil *	Male	1.82	1.36	0.00-4.15
(%)	Female	0.92	1.04	0.00-3.59
	Both sexes	1.38	1.28	0.00-4.15
Differential eosinophil	Male	9.05	2.55	3.52-13.57
(%)	Female	10.92	4.16	5.58-20.50
	Both sexes	9.95	3.50	3.52-20.50
Differential basophil	Male	0.77	0.72	0.00-2.04
(%)	Female	0.41	0.42	0.00-1.03
	Both sexes	0.60	0.61	0.00-2.04

Remarks: [a] Data are derived from 17 male, 16 female or both (33 male and female) frogs.

[*] Significant difference of mean parameter values between males and females, Student's *t*-test ($P \leq 0.05$)

Discussions

Data on the hematological parameters of wild amphibians in Asia is still limited. This study is the first report of the hematological parameters in wild caught *H. rugulosus* in Southeast Asia. The number of erythrocytes, as determined by hemocytometer counts, in *H. rugulosus* was higher than that previously reported for other anurans, including that of a closely related species, *R. tigrina* (Singh, 1977a).

The presence of immature erythrocytes in the circulating blood can occur under normal (healthy) conditions in vertebrates, but if this reaches above the normal value, it indicates stress-related condition in erythropoiesis (Briggs and Bain, 2012). In general, an abnormal increase in the number of immature erythrocytes in the circulating blood indicates a compensatory regenerative response due to anemia or loss of circulating erythrocytes (Allender and Fry, 2008) or infectious diseases (Campbell, 2015). Because of the lack of baseline data of circulating immature erythrocyte levels in *H. rugulosus*, it was not possible to determine if these are normal or abnormal levels of circulating immature erythrocytes, but the external appearance of the frogs were normal and healthy. Mitosis of erythrocytes in peripheral blood were reported in amphibians (Dawson, 1931) and frequently found in reptiles (Campbell, 2015; Salakij, 2005). They were commonly seen as the erythrocyte containing mitotic figure which was also evidenced in this study. Since it is known that the circulating erythrocytes are in genetically dormant state meaning they do not undergo mitosis (Orr et al., 1986), it can be assumed that these mitotic cells are from immature erythrocytes which were reported to retain this ability (Thomas and Maclean, 1975).

Sex-related differences in some blood parameters are known to exist in mammals and other vertebrates (Golemi et al., 2013; Nemeth et al., 2010; Xie et al., 2013). From the blood smear examinations of *H. rugulosus*, the differential lymphocyte and neutrophil counts showed sex-related difference with higher values in males. However, very few researches have reported sex-related differences of these parameters previously in amphibians. A study in wild-caught Indian tree frog (*Polypedates maculatus*) reported significantly higher monocyte and eosinophil counts in males than in females (Mahapatra et al., 2012), while the neutrophil/lymphocyte ratio was significantly higher in wild-caught female mole

salamanders (*Ambystoma talpoideum*), which was attributed to reproductive stress (Davis and Maerz, 2008). Given that the sampling period used in this study was with the reproductive period of *H. rugulosus*, this may have been the cause of the observed sex-related differences in the blood parameters detected in this study.

From the differential leukocyte counts, agranulocytes were found to comprise a seven-fold higher proportion of the leukocytes than granulocytes (88% and 12% of all leukocytes, respectively). The most abundant leukocytes in *H. rugulosus* peripheral blood were lymphocytes, which is similar to previous reports in other frog species (Arikan and Cicek, 2014; Cabagna et al., 2005; Cathers et al., 1997; Das and Mahapatra, 2012; Singh, 1977b).

The PCV of these *H. rugulosus* also showed a significant sex-related difference with a higher PCV value in males. The PCV value has been reported to indicate the erythrocyte mass in amphibians (Allender and Fry, 2008). However, the mean PCV value of *H. rugulosus* regardless of the gender (28%) was similar to those reported in other frogs (Cabagna et al., 2005; Cathers et al., 1997; Donmez et al., 2009; Gul et al., 2011; Mahapatra et al., 2012; Sinha, 1983; Wojtaszek and Adamowicz, 2003), but lower than those of salamander (Solis et al., 2007). The difference in PCV values among amphibians is believed to depend on differences in sex, season, habitat and natural history of each species.

The plasma total protein of male *H. rugulosus* was higher, but not significantly, than that of female. This result is similar to the previous reports in european green toad (*Pseudepidalea viridis*), eastern spadefoot toad (*Pseudepidalea viridis*) and european tree frog (*Hyla arborea*) (Gul et al., 2011).

The morphology of blood cells in amphibians is similar in terms of their general characteristics such as cell shape, nuclear shape and granules in cytoplasm. But some specific characteristics may vary among species, especially the size of each cell type. In this study, the erythrocyte size, determined as dimensions and area, fell within the ranges reported for other anurans (Arıkan and Cicek, 2010). The significant difference between the nucleocytoplasmic ratios of circulating mature and immature erythrocytes in *H. rugulosus* confirmed the identification criteria of immature cells,

where a higher nucleocytoplasmic ratio indicates less mature cells. The mean nucleocytoplasmic ratio of circulating erythrocytes in these *H. rugulosus* was in the range reported in other anurans (Arikan and Cicek, 2014). However, the mean length and width of thrombocytes in these *H. rugulosus* suggested that this cell type was more ellipsoid than other anurans, while leukocytes also showed a difference in size (diameter) of each leukocyte type compared with that in other anuran species (Arıkan and Cicek, 2010). The largest circulating leukocytes in *H. rugulosus* were basophils and the smallest were lymphocytes.

Conclusion

Based on their morphological characteristics, cells in the peripheral blood of *H. rugulosus* were classified into mature and immature erythrocytes, leukocytes (lymphocytes, monocytes, neutrophils, basophils and eosinophils) and thrombocytes. The presence of immature erythrocytes, even though frequently reported in vertebrates, is interesting since an increase in their circulating level can indicate a disturbance of the erythron. Thus, the data obtained in this study will be useful in assessing abnormality in the erythron of *H. rugulosus* in the future. Moreover, these results revealed that the PCV and the differential lymphocyte and neutrophil counts of male frogs were significantly higher than those of the females. However, the size of each blood cell type was not significantly different between sexes of *H. rugulosus*. The hematological parameters presented in this study are the first report for *H. rugulosus* and so represent the crucial baseline data for wild *H. rugulosus* in Thailand that can be expanded upon to use for monitoring the health status of this anuran in the future.

CHAPTER IV
HEMATOLOGICAL PARAMETERS AND THE NORMAL REFERENCE VALUES OF FARM RAISED RICE FIELD FROG *Hoplobatrachus rugulosus*

Introduction

The rice field frog, *Hoplobatrachus rugulosus* (Wiegmann, 1834) is a well-known species of anuran amphibian in the family Dicroglossidae. It distributes in wetlands and paddy fields throughout central China and Myanmar to Thailand and peninsular Malaysia (Diesmos et al., 2004). This frog is an economically important animal of Thailand. It also contributes to economics of other countries because it is food of the local people. As a result, frog farming is increasing and becomes more popular in response to high market demand. Like other farmed animals, farmed frogs often have stress and diseases due to limitation of space. Currently, health status monitoring of *H. rugulosus* in farm is generally based on visual inspection because there is no report about normal value of hematological parameters of this frog that can be used to assess the overall health status and monitor the spread of disease in the farming ponds. Therefore, the hematological parameters that can effectively be used to evaluate the health status will be a solution to reduce the risk of disease spreading in the farm raised frogs.

Hematological technique is a basic method to determine health of animal, because this method is relatively rapid and easy compared with other methods. The result from hematological study could indicate basic animal health status. It has been reported that changes of hematological parameters can indicate stress (Davis et al., 2008) and inception of disease (Burgmeier et al., 2011). Hematological parameters that frequently been used to assess the health of animals are packed cell volume (PCV) or hematocrit, erythrocyte count, leukocyte count and differential leukocyte count. These parameters are important fundamental to health assessment in animals. Data on hematological parameters of human and domestic mammals are well documented, but for amphibian, it is still limited. Some papers have reported the

characteristics of amphibian blood (Bricker et al., 2012; Canfield, 1998; Claver and Quaglia, 2009; Das and Mahapatra, 2012). Most of them studied on hematology in various species of *Rana* (Davis et al., 2008; Omonona and Ekpenko, 2011; Palenske and Saunders, 2003; Sinha, 1983; Weathers, 1975). However, data on reference hematological values were rarely reported in amphibian species.

Hematological normal reference value is a set of value resulting from blood sample test of healthy animals, that used by a health professional to interpret a set of medical test results. It is a basis for a physician to interpret a set of test results for a particular domestic animal and human patient. The hematological test result that falls out of the normal reference value can indicate abnormality of the patient's health status and it is needed for further advanced investigation. A normal reference value of a hematological parameter is usually defined as the set of values that 95 percent of the normal population falls within, that is 95% prediction interval (Bain et al., 2012). This hematological reference value is very important and essential to establish the diagnostic criteria in every population to ensure appropriate interpretation of results in health assessments.

The hematological parameters as well as its normal reference values can be varied considerably under the influence of different factors such as the age and sex, and various environmental factors such as seasons (Bain et al., 2012). So, the objective of this study is to determine hematological parameters of farm raised *H. rugulosus* in different sexes, age groups and seasons; and to estimate the hematological normal reference values of farm raised *H. rugulosus*. The data obtained will be useful for the health assessment of this farm raised frog species in the future.

Materials and Methods

1. Study site and animal collection

1.1 Study site

Farm raised *H. rugulosus* was obtained from the Huai Hong Khrai Royal Development Study Center (UTM 523200 2807200, zone line 47Q), Chiang Mai Province, northern Thailand. The frog farm is located in plain-foothills inside this

center. This farm is operated under supervision of expert officials in order to provide an example of standardized frog farm system for local people. The farm contains cement ponds (in the size of 2.0×2.5×1.2 to 3.0×4.0×1.2 m) with covered roof (Figure 4-1). The environmental factors such as light and temperature are natural. The density of the frogs in each pond at the study site was in an appropriate proportion. At tadpole stage, the density was 100-500 tadpoles/m^2. At juvenile stage, the density was 100-300 frogs/m^2. At adult stage, the density was 50-80 frogs/m^2. The frog ponds were cleaned and the water in each pond was changed everyday. In each pond the dry surface substrates were provided for the frogs to rest. Fish pellets were used as food for feeding the frogs once a day. Natural foods including earthworms, termites and other insects which would be protein supplements were also given to the frogs.

Figure 4-1 The study site at the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province

1.2 Sampling seasons

The samplings of farm raised *H. rugulosus* were done once in each season in three seasons within one year. The three sampling periods were February 2014 (cool dry season), May 2014 (hot dry season) and August 2014 (wet season). The seasons were defined based on a climograph plotted between mean values of temperature and total rainfall (Walter et al., 1975). The climograph indicated that the dry season ranged from January (cool dry) to June (hot dry) in 2014 while the wet season ranged from July to October in 2014. The average temperatures of the sampling periods were 24.3 °C in cool dry season, 29.1 °C in hot dry season and 27.4 °C in wet season.

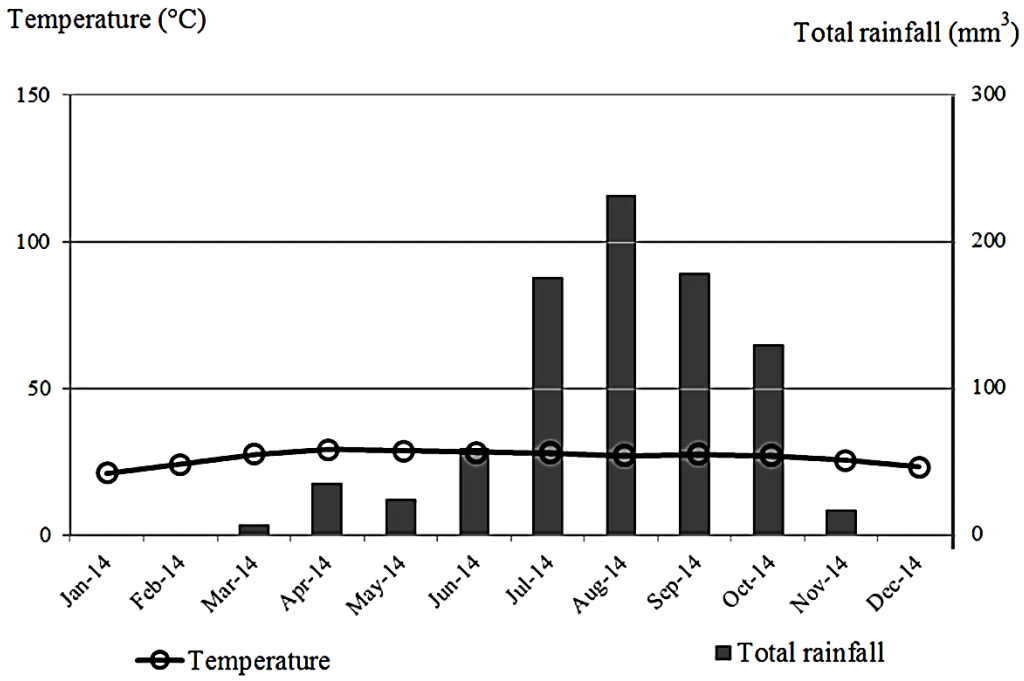

Figure 4-2 Climograph of the study site at the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province, in 2014

1.3 Frog collection and blood sampling

In each season, 40 adult (male = 20, female = 20) and 40 juvenile (male = 20, female = 20) *H. rugulosus* with healthy external morphology were collected from the study site. The frogs were anesthetized in ice slurry. Snout-vent length (SVL) and body weight (BW) of each frog were recorded. Blood sample (0.5 mL per 100 g body weight) was collected from each frog by cardiac puncture (Heatley and Johnson, 2009) using 25-gauge needle with heparinized tuberculin syringe and transferred to a microcentrifuge tube. The samples were stored in ice bucket during an hour of PCV, hemocytometer counting and blood smear processes.

2. Hematological parameters

2.1 Erythrocyte and leukocyte counts

In this study, erythrocyte and leukocyte counts were determined manually using a hemocytometer (Tharp and Woodman, 2002). In each season, only 10 adult (male = 5, female = 5) and 10 juvenile (male = 5, female = 5) *H. rugulosus* were sampled for blood for erythrocyte and leukocyte hemocytometer counting. The blood sample from each frog was primarily diluted small amount of with Natt and Herrick's solution (Natt and Herrick, 1951) in diluting pipette. Then, the Natt and Herrick's solution (Appendix B-II) was taken continuously with the same pipette that already contained blood sample, until the mixed solution reaching the scale marked 101 (for erythrocyte count) or 11 (for leukocyte count). The pipette was rotated gently until the blood sample and the solution was well mixed. The solution inside was 1:200 dilution of blood sample in the Natt and Herrick's solution. Then, a cover slip was placed in the position that covered the Neubauer ruling area of the counting chamber. The pressure was slightly applied on a rubber tube that connected with the pipette so that a drop of blood solution was in hanging position. The tip of the pipette (with a hanging drop) was positioned to touch against the edge of the cover slip on the counting chamber. Then, the chamber was getting filled with the blood solution. During this process, air bubble should not present inside the chamber and the blood solution should not over filled on the ruling area. The counting chamber was left without disturbance about 3 minutes. After that, the chamber was placed on the stage

of a light microscope. Blood counting was performed using light microscope at 400× magnification. The total number of viable cells/µL was obtained by the following equation.

Total number of viable erythrocyte cells/µL	=	average viable erythrocyte cell count	×	5,000
Total number of viable leukocyte cells/µL	=	average viable leukocyte cell count	×	1,000

2.2 Packed cell volume

The blood sample of each frog was transferred to a microcapillary tube. The blood-filled microcapillary tube was sealed at one end with Critoseal wax before placing in a microhematocrit centrifuge (Centurion Scientific LTD, *1020 series*). Then, the samples were centrifuged at 8,700 ×g for 10 minutes (Salakij, 2005). After centrifugation, the microcapillary tubes were placed in a microhematocrit reader to evaluate the hematocrit value according to the manufacturer's instruction on the microhematocrit reading device.

2.3 Differential leukocyte count

The Giemsa stained blood smear slides were examined using 400× magnification under a light microscope in the areas that the cell morphology was normal and the cells were well-dispersed. A total of 200 cells were counted in a strip running manner adapted from a straight-edge method (Salakij, 2005) for each leukocyte type including lymphocyte, monocyte, neutrophil, basophil and eosinophil. Then the percentage of each cell type was calculated.

2.4 Plasma total protein determination

The blood sample (0.5 - 0.7mL) of each frog had been transferred to a microcentrifuge tube and centrifuged at 1,500 ×g at 4°C for 15 minutes. The plasma separated by centrifugation was collected and transferred into a new microcentrifuge tube and stored at -20°C. The plasma samples were subjected to the procedure of total protein determination by Bradford assay (Bradford, 1976). The samples were analyzed in a 96-well plate using a stock solution of 25 µg/mL bovine serum albumin

(BSA) as a standard protein. The BSA standard and the plasma samples were diluted with phosphate buffered saline (PBS; Appendix B-I). For BSA standard, serial dilution was performed to obtain the concentration from 0.78125 to 25 µg/mL. The plasma samples were diluted with PBS at 1:1,000 and 1:10,000 dilutions. One hundred microliters of standards, quality control sample (QC; BSA 10 µg/mL), blank (PBS) and samples were added in duplicate into each well. A 100 µL aliquot of Bradford solution (Sigma) was added into each well using a muti-channel pipette. The microplate filled with samples was shaken with PSU 2-T mini shaker for a few minutes at room temperature before measuring for absorbance by Multiskan EX microplate reader at 595 nm (Redinbaugh and Campbell, 1985). The average absorbance of each standard was corrected by subtracting with an average absorbance of PBS blank. A standard calibration curve was constructed by plotting the corrected absorbance on a vertical (Y) axis versus the corresponding BSA concentration on horizontal (X) axis followed by linear regression analysis using Microsoft Excel software (Microsoft Corporation, Redmond, USA). The plasma protein concentration of the samples were determined using linear regression equation of the standard curve.

3. Statistical Analyses

All data of each blood parameter were tested for normal distribution by Kolmogorov-Smirnov test and homogeneity of variance. The hematological parameters were compared between sexes by Student *t*-test for the data that are normally distributed or Mann-Whitney rank sum test for the data that are not normally distributed. All parameters were compared among seasons by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls methods for the data that are normally distributed. If the data are not normally distributed, all parameters were compared between seasons by Kruskal-Wallis Analysis of Variance on Ranks followed by Dunn's Method (Zar, 1998). Sigma Plot version 11.0 software (Systat Software, San Jose, USA) was used as statistical software for every tests.

All blood parameters defined for normal reference values were tested for normal distribution by Kolmogorov-Smirnov test. If the data are not normally distributed, the data were ran through the Box-Cox power transformations before

being analyzed by Reference Value Advisor V2.1 software (National Veterinary School, Toulouse, France; Geffre et al., 2011). Outliers were deleted if the difference between the outlying value and the adjacent value exceeded one third of the total range of all values. Values over three-times of the standard deviation (SD) were also deleted (Solberg, 1999; Yu et al., 2013). When the data were from normal distribution, reference values were defined by minimum and maximum values ($n \leq 40$) or mean ± 2SD and the 95% confident limits ($n > 40$; Solberg, 1999).

Results

1. Hematological parameters

1.1 Sex-related difference of hematological parameters

All hematological parameters of peripheral blood of farm raised *H. rugulosus* were recorded separately between sexes, adult/juvenile and seasons (cool dry, hot dry, wet). For each parameter in each season, the data between adult males and adult females as well as the data between juvenile males and juvenile females were statistically analyzed for sex-related difference. In case that the significant difference was presented, the data were consequently analyzed separately according to sex.

The significant differences between sexes of the hematological parameters of adult and juvenile *H. rugulosus* are summarized in Table 4-1. The hematological parameters of adult frogs that showed significant sex-related difference (Student *t*-test, $p > 0.05$) include erythrocyte count, packed cell volume, plasma total protein, differential lymphocyte count, differential monocyte count and differential eosinophil count. The hematological parameters of juvenile frogs that showed significant sex-related difference (Student *t*-test, $p > 0.05$) include packed cell volume, plasma total protein, differential lymphocyte count, differential monocyte count, differential neutrophil count and differential eosinophil count. All these sex-related different parameters were analyzed further in comparison between seasons separately between males and females. The parameters without sex-related difference were analyzed in comparison among seasons as one group of both males and females.

Table 4-1 Sex-related difference in hematological parameters of adult and juvenile *H. rugulosus*, collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in 2014

Parameters	Sex-related significant difference					
	Cool dry season		Hot dry season		Wet season	
	Adult	Juvenile	Adult	Juvenile	Adult	Juvenile
Erythrocyte count	-	-	✓	-	-	-
Leukocyte count	-	-	-	-	-	-
Packed cell volume (PCV)	-	-	✓	✓	✓	✓
Plasma total protein	✓	✓	-	-	-	-
Differential lymphocyte count	-	-	✓	-	-	✓
Differential monocyte count	-	✓	✓	-	-	-
Differential neutrophil count	-	-	-	-	-	✓
Differential basophil count	-	-	-	-	-	-
Differential eosinophil count	✓	-	-	-	-	✓

Remark: Significant sex-related difference (Student *t*-test, $p < 0.05$) is indicated by a check mark (✓).

1.2 Seasonal difference of hematological parameters

1.2.1 Erythrocyte count

The values of erythrocyte count of peripheral blood of *H. rugulosus* are summarized in Table 4-2 and Figure 4-3. In cool dry season, the mean values of erythrocyte count of adult *H. rugulosus* were 0.63 ± 0.25 $\times 10^6$ and 0.86 ± 0.47 $\times 10^6$ cells/µL in males and females, respectively. The mean value of erythrocyte count of juvenile *H. rugulosus* was 0.82 ± 0.39 $\times 10^6$ cells/µL. In hot dry season, the mean values of erythrocyte count of adult *H. rugulosus* were 1.05 ± 0.30 $\times 10^6$ and 0.65 ± 0.22 $\times 10^6$ cells/µL in males and females, respectively. The mean value of erythrocyte count of juvenile *H. rugulosus* was 0.93 ± 0.32 $\times 10^6$ cells/µL. In wet season, the mean values of erythrocyte count of adult *H. rugulosus* were 1.23 ± 0.65 $\times 10^6$ and 1.68 ± 0.28 $\times 10^6$ cells/µL in males and females, respectively. The mean value of erythrocyte count of juvenile *H. rugulosus* was 1.49 ± 0.51 $\times 10^6$ cells/µL.

After seasonal variation analysis (Table 4-2, Figure 4-3), the result showed that in wet season, the mean values of erythrocyte count of adult female *H. rugulosus* was significantly higher than those of the females in cool dry and hot dry seasons (one-way ANOVA, F = 12.93, df = 2, $P = 0.001$). The mean value of erythrocyte count of juveniles in wet season was also significantly higher than those of the juveniles in cool dry and hot dry seasons (one-way ANOVA, F = 9.21, df = 2, $P < 0.001$).

Table 4-2 Erythrocyte count values (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Erythrocyte counts ($\times 10^6$ cells/µL)		
	Adult		Juvenile
	Males (n=5)	Females (n=5)	Males & Females (n=5)
Cool dry	0.63 ± 0.25	0.86 ± 0.47 ^{b}	0.82 ± 0.39 ^{B}
Hot dry	1.05 ± 0.30	0.65 ± 0.22 ^{b}	0.93 ± 0.32 ^{B}
Wet	1.23 ± 0.65	1.68 ± 0.28 ^{a}	1.49 ± 0.51 ^{A}

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult females and capital letters for juvenile frogs).

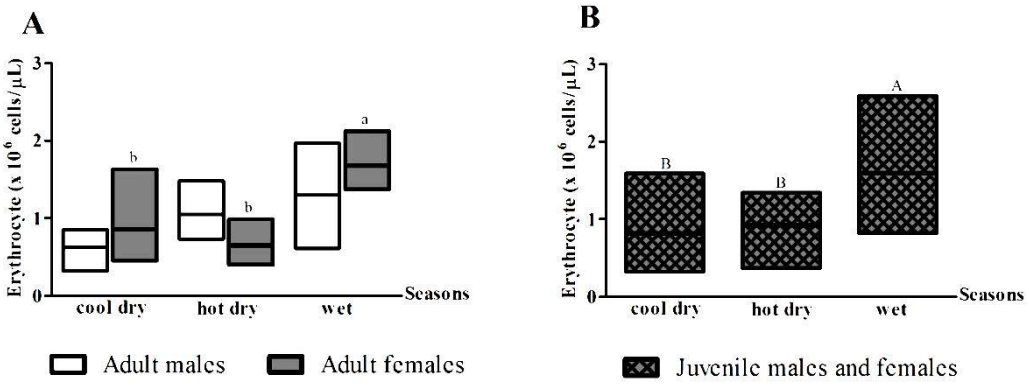

Figure 4-3 Erythrocyte count values (mean and range) of **(A)** adult and **(B)** juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult males and capital letters for juvenile frogs).

1.2.2 Leukocyte count

The values of leukocyte count of peripheral blood of *H. rugulosus* are summarized in Table 4-3 and Figure 4-4. In cool dry season, the mean value of leukocyte count of adult *H. rugulosus* was 3.88 ± 1.95 $\times 10^3$ cells/µL. The mean value of leukocyte count of juvenile *H. rugulosus* was 3.74 ± 1.70 $\times 10^3$ cells/µL. In hot dry season, the mean value of leukocyte count of adult *H. rugulosus* was 6.30 ± 3.01 $\times 10^3$ cells/µL. The mean value of leukocyte count of juvenile *H. rugulosus* was 4.62 ± 3.86 $\times 10^3$ cells/µL. In wet season, the mean value of leukocyte count of adult *H. rugulosus* was 7.32 ± 2.92 $\times 10^3$ cells/µL. The mean value of leukocyte count of juvenile *H. rugulosus* was 6.53 ± 1.44 $\times 10^3$ cells/µL.

After seasonal variation analysis (Table 4-3, Figure 4-4), the result showed that in wet season, the mean values of leukocyte count of adult *H. rugulosus* was significantly higher than those of the frogs in cool dry and hot dry seasons (one-way ANOVA, F = 4.37, df = 2, P = 0.023). The mean value of leukocyte count of juveniles in wet season was also significantly higher than those of the frogs in cool dry and hot dry seasons (one-way ANOVA, H = 10.35, df = 2, P = 0.006).

Table 4-3 Leukocyte count values (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Leukocyte count ($\times 10^3$ cells/µL)	
	Adult	Juvenile
	Males & Females (n=10)	Males & Females (n=10)
Cool dry	3.88 ± 1.95 ^{b}	3.74 ± 1.70 ^{B}
Hot dry	6.30 ± 3.01 ^{b}	4.62 ± 3.86 ^{B}
Wet	7.32 ± 2.92 ^{a}	6.53 ± 1.44 ^{A}

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult and capital letters for juvenile frogs).

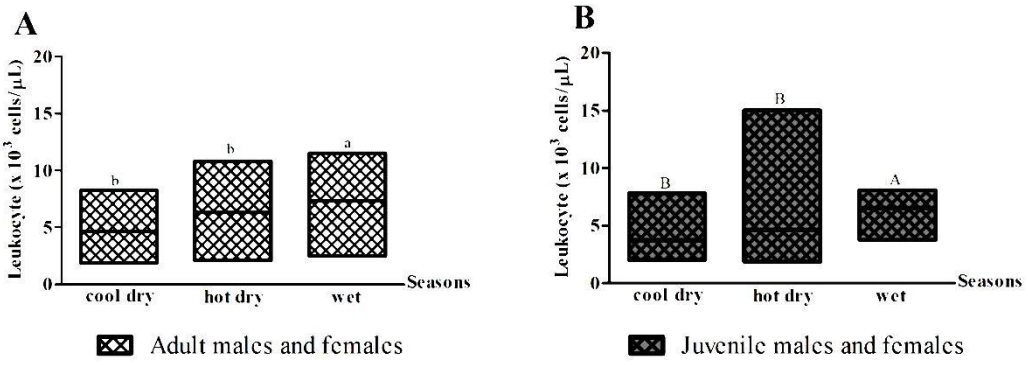

Figure 4-4 Leukocyte count values (mean and range) of **(A)** adult and **(B)** juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult and capital letters for juvenile frogs).

1.2.3 Packed cell volume (PCV)

The values of PCV of peripheral blood of *H. rugulosus* are summarized in Table 4-4 and Figure 4-5. In cool dry season, the mean values of PCV count of adult *H. rugulosus* were 36.45 ± 7.03 and 34.55 ± 8.24 % in males and females, respectively. The mean values of PCV of juvenile *H. rugulosus* were 39.18 ± 6.00 and 37.50 ± 8.17 % in males and females, respectively. In hot dry season, the mean values of PCV of adult *H. rugulosus* were 47.13 ± 5.24 and 36.63 ± 4.53 % in males and females, respectively. The mean values of PCV of juvenile *H. rugulosus* were 42.38 ± 4.84 and 35.45 ± 5.17 % in males and females, respectively. In wet season, the mean values of PCV of adult *H. rugulosus* were 41.43 ±7 .45 and 36.90 ± 5.79 % in males and females, respectively. The mean values of PCV of juvenile *H. rugulosus* were 43.30 ± 3.12 and 38.30 ± 3.71 % in males and females, respectively.

After seasonal variation analysis (Table 4-4, Figure 4-5), the result showed that in hot dry season, the mean value of PCV of adult male *H. rugulosus* was significantly higher than those of the males in cool dry and wet seasons (one-way ANOVA, H = 20.00, df = 2, P = < 0.001). The mean value of PCV of juvenile males in cool dry season was significantly lower than those of the males in hot dry and wet seasons (one-way ANOVA, H = 7.175, df = 2, P = 0.028).

Table 4-4 PCV values (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	PCV (%)			
	Adult		Juvenile	
	Males (n=20)	Females (n=20)	Males (n=20)	Females (n=20)
Cool dry	36.45 ± 7.03 [b]	34.55 ± 8.24	39.18 ± 6.00 [B]	37.50 ± 8.17
Hot dry	47.13 ± 5.24 [a]	36.63 ± 4.53	42.38 ± 4.84 [A]	35.45 ± 5.17
Wet	41.43 ± 7.45 [b]	36.90 ± 5.79	43.30 ± 3.12 [A]	38.30 ± 3.71

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult males and capital letters for juvenile males).

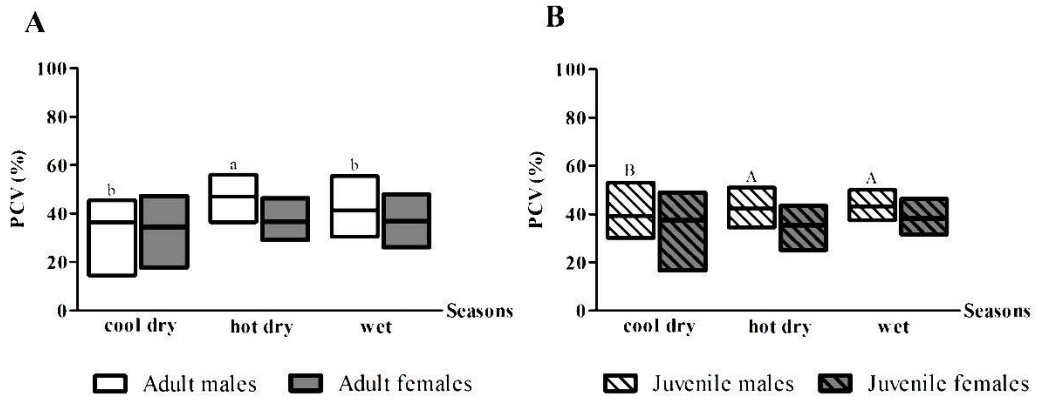

Figure 4-5 PCV values (mean and range) of **(A)** adult and **(B)** juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult males and capital letters for juvenile males).

1.2.4 Plasma total protein

The values of plasma total protein of peripheral blood of farm raised *H. rugulosus* are summarized in Table 4-5 and Figure 4-6. In cool dry season, the mean values of plasma total protein of adult *H. rugulosus* were 64.95 ± 11.10 and 86.28 ± 13.92 mg/mL in males and females, respectively. The mean values of plasma total protein of juvenile *H. rugulosus* were 56.28 ± 24.75 and 70.39 ± 25.70 mg/mL in males and females, respectively. In hot dry season, the mean values of plasma total protein of adult *H. rugulosus* were 52.75 ± 13.28 and 49.18 ± 9.71 mg/mL in males and females, respectively. The mean values of plasma total protein of juvenile *H. rugulosus* were 47.62 ± 8.80 and 53.50 ± 10.51 mg/mL in males and females, respectively. In wet season, the mean values of plasma total protein of adult *H. rugulosus* were 56.88 ± 15.58 and 56.66 ± 14.28 mg/mL in males and females, respectively. The mean values of plasma total protein of juvenile *H. rugulosus* were 70.47 ± 10.75 and 65.87 ± 8.85 mg/mL in males and females, respectively.

After seasonal variation analysis (Table 4-5, Figure 4-6), the result showed that in cool dry season, the mean value of plasma total protein of adult male *H. rugulosus* was significantly higher than that of the males in hot dry season (one-way ANOVA, F = 4.26, df = 2, $P = 0.019$), but not significantly different from that of the males in wet season (one-way ANOVA, F = 4.26, df = 2, $P = 0.149$). The mean value of plasma total protein in cool dry season of adult female *H. rugulosus* was also significantly higher than those of the females in hot dry and wet seasons (one-way ANOVA, H = 35.54, df = 2, $P = < 0.001$). In hot dry season, the mean value of plasma total protein of juvenile female *H. rugulosus* was significantly lower than those of the females in cool dry and wet seasons (one-way ANOVA, H = 11.61, df = 2, $P = 0.003$). In wet season, the mean value of plasma total protein of juvenile male *H. rugulosus* was significantly higher than those of the females in cool dry and hot dry seasons (one-way ANOVA, H = 21.68, df = 2, $P = < 0.001$).

Table 4-5 Plasma total protein values (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Plasma total protein (mg/mL)			
	Adult		Juvenile	
	Males (n=20)	Females (n=20)	Males (n=20)	Females (n=20)
Cool dry	64.95 ± 11.10 [a]	86.28 ± 13.92 [A]	56.28 ± 24.75 [b]	70.39 ± 25.70 [A]
Hot dry	52.75 ± 13.28 [b]	49.18 ± 9.71 [B]	47.62 ± 8.80 [b]	53.50 ± 10.51 [B]
Wet	56.88 ± 15.58 [ab]	56.66 ± 14.28 [B]	70.47 ± 10.75 [a]	65.87 ± 8.85 [A]

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for males and capital letters for females).

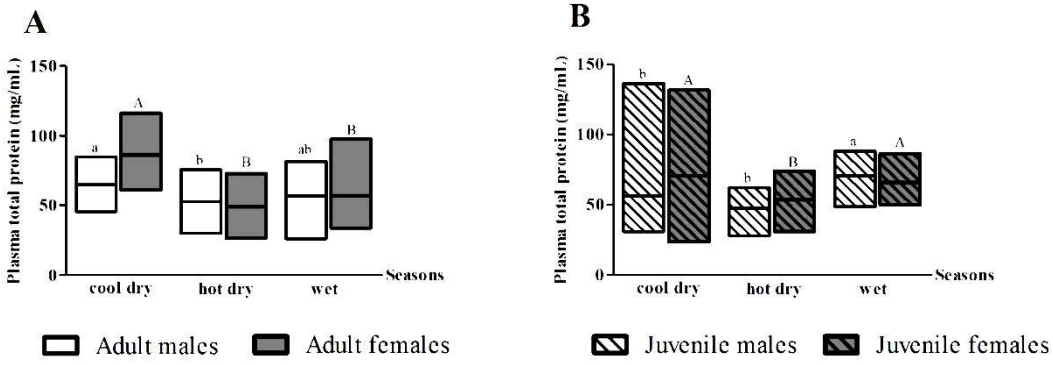

Figure 4-6 Plasma total protein values (mean and range) of **(A)** adult and **(B)** juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for males and capital letters for females).

1.2.5 Differential leukocyte counts

1.2.5.1 Differential lymphocyte count

The values of differential lymphocyte count of peripheral blood of *H. rugulosus* are summarized in Table 4-6 and Figure 4-7. In cool dry season, the mean values of differential lymphocyte count of adult *H. rugulosus* were 54.90 ± 7.53 and 56.22 ± 8.68 % in males and females, respectively. The mean values of differential lymphocyte count of juvenile *H. rugulosus* were 59.75 ± 6.50 and 57.05 ± 5.63 % in males and females, respectively. In hot dry season, the mean values of differential lymphocyte count of adult *H. rugulosus* were 57.44 ± 5.43 and 66.95 ± 10.44 % in males and females, respectively. The mean values of differential lymphocyte count of juvenile *H. rugulosus* were 54.86 ± 5.26 and 55.70 ± 4.29 % in males and females, respectively. In wet season, the mean values of differential lymphocyte count of adult *H. rugulosus* were 70.55 ± 7.27 and 72.44 ± 4.99 % in males and females, respectively. The mean values of differential lymphocyte count of juvenile *H. rugulosus* were 60.43 ± 6.01 and 67.19 ± 6.07 % in males and females, respectively.

After seasonal variation analysis (Table 4-6, Figure 4-7), the result showed that the mean values of differential lymphocyte count of adult female *H. rugulosus* were significantly different among all seasons (one-way ANOVA, H = 23.69, df = 2, P = < 0.001). In wet season, the mean value of differential lymphocyte count of adult male *H. rugulosus* was significantly higher than those of the males in cool dry and hot dry seasons (one-way ANOVA, F = 30.77, df = 2, P < 0.001). In hot dry season, the mean value of differential lymphocyte count of juvenile male *H. rugulosus* was significantly lower than those of the males in cool dry and wet seasons (one-way ANOVA, H = 8.62, df = 2, P = 0.013). The mean values of differential lymphocyte count in wet season of juvenile female *H. rugulosus* was also significantly higher those of the females in cool dry and hot dry seasons (one-way ANOVA, F = 27.22, df = 2, P < 0.001).

Table 4-6 Differential lymphocyte counts (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Differential lymphocyte counts (%)			
	Adult		Juvenile	
	Males (n=20)	Females (n=20)	Males (n=20)	Females (n=20)
Cool dry	54.90 ± 7.53 ^{b}	56.22 ± 8.68 ^{C}	59.75 ± 6.50 ^{a}	57.05 ± 5.63 ^{B}
Hot dry	57.44 ± 5.43 ^{b}	66.95 ± 10.44 ^{B}	54.86 ± 5.26 ^{b}	55.70 ± 4.29 ^{B}
Wet	70.55 ± 7.27 ^{a}	72.44 ± 4.99 ^{A}	60.43 ± 6.01 ^{a}	67.19 ± 6.07 ^{A}

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for males and capital letters for females).

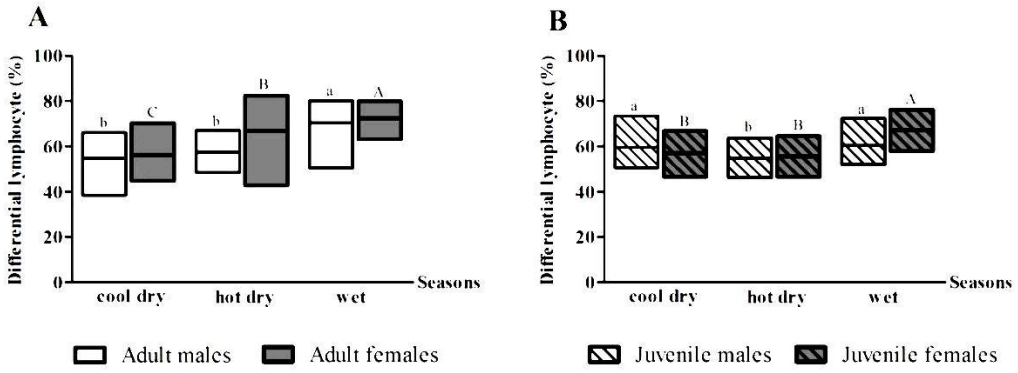

Figure 4-7 Differential lymphocyte counts (mean and range) of **(A)** adult and **(B)** juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for males and capital letters for females).

1.2.5.2 Differential monocyte count

The values of differential monocyte count of peripheral blood of *H. rugulosus* are summarized in Table 4-7 and Figure 4-8. In cool dry season, the mean values of differential monocyte count of adult *H. rugulosus* were 35.30 ± 7.29 and 37.22 ± 7.81 % in males and females, respectively. The mean value of differential monocyte count of juvenile *H. rugulosus* was 32.46 ± 5.26 %. In hot dry season, the mean values of differential monocyte count of adult *H. rugulosus* were 34.12 ± 4.72 and 24.18 ± 9.87 % in males and females, respectively. The mean value of differential monocyte count of juvenile *H. rugulosus* was 33.95 ± 4.30 %. In wet season, the mean values of differential monocyte count of adult *H. rugulosus* were 20.18 ± 5.62 and 19.06 ± 5.62 % in males and females, respectively. The mean value of differential monocyte count of juvenile *H. rugulosus* was 25.29 ± 5.78 %.

After seasonal variation analysis (Table 4-7, Figure 4-8), the result showed that the mean values of differential monocyte count of adult female *H. rugulosus* were significantly different among all seasons (one-way ANOVA, $F = 27.70$, $df = 2$, $P < 0.001$). In wet season, the mean values of differential monocyte count of adult male *H. rugulosus* was significantly lower than those of the males in cool dry and hot dry seasons (one-way ANOVA, $F = 39.66$, $df = 2$, $P < 0.001$). The mean value of differential monocyte count in wet season of juvenile *H. rugulosus* was also significantly lower than those of the juveniles in cool dry and hot dry seasons (one-way ANOVA, $F = 32.37$, $df = 2$, $P < 0.001$).

Table 4-7 Differential monocyte counts (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Differential monocyte counts (%)		
	Adult		Juvenile
	Males (n=20)	Females (n=20)	Males & Females (n=40)
Cool dry	35.30 ± 7.29 [a]	37.22 ± 7.81 [A]	32.46 ± 5.26 [I]
Hot dry	34.12 ± 4.72 [a]	24.18 ± 9.87 [B]	33.95 ± 4.30 [I]
Wet	20.18 ± 5.62 [b]	19.06 ± 5.62 [C]	25.29 ± 5.78 [II]

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult males and capital letters for adult females) and roman numerals for juvenile frogs.

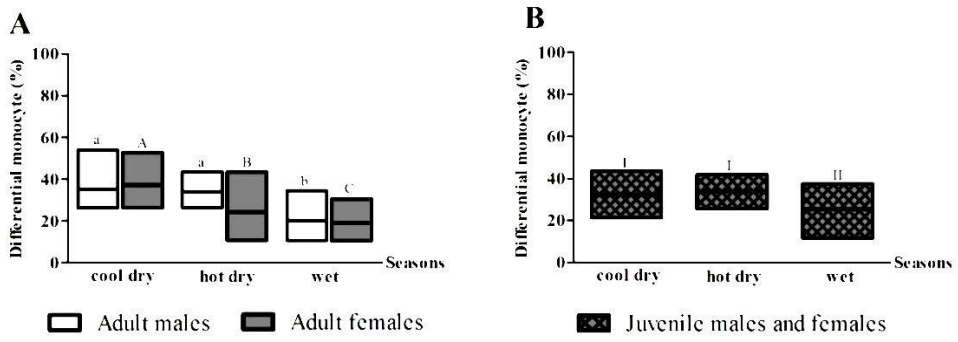

Figure 4-8 Differential monocyte counts (mean and range) of **(A)** adult and **(B)** juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult males and capital letters for adult females) and roman numerals for juvenile frogs.

1.2.5.3 Differential neutrophil count

The values of differential neotrophil count of peripheral blood of *H. rugulosus* are summarized in Table 4-8 and Figure 4-9. In cool dry season, the mean value of differential neutrophil count of adult *H. rugulosus* was 0.34 ± 0.57 %. The mean values of differential neutrophil count of juvenile *H. rugulosus* were 0.73 ± 0.66 and 0.85 ± 0.65 % in males and females, respectively. In hot dry season, the mean value of differential neutrophil count of adult *H. rugulosus* was 0.57 ± 0.59 %. The mean values of differential neutrophil count of juvenile *H. rugulosus* were 0.66 ± 0.52 and 0.73 ± 0.70% in males and females, respectively. In wet season, the mean value of differential neutrophil count of adult *H. rugulosus* was 0.23 ± 0.32 %. The mean values of differential neutrophil count of juvenile *H. rugulosus* were 0.63 ± 0.69 and 0.25 ± 0.35% in males and females, respectively.

After seasonal variation analysis (Table 4-8, Figure 4-9), the result showed that in hot dry season, the mean value of differential neutrophil count of adults *H. rugulosus* was significantly lower than those of the frogs in cool dry and wet seasons (one-way ANOVA, H = 7.70, df = 2, P = 0.021). In wet season, the mean values of differential neutrophil count of juvenile female *H. rugulosus* was significantly lower than those of the females in cool dry and hot dry seasons (one-way ANOVA, H = 11.96, df = 2, P = 0.003).

Table 4-8 Differential neutrophil counts (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Differential neutrophil counts (%)		
	Adult	Juvenile	
	Males & Females (n=40)	Males (n=20)	Females (n=20)
Cool dry	0.34 ± 0.57 [b]	0.73 ± 0.66	0.85 ± 0.65 [A]
Hot dry	0.57 ± 0.59 [a]	0.66 ± 0.52	0.73 ± 0.70 [A]
Wet	0.23 ± 0.32 [b]	0.63 ± 0.69	0.25 ± 0.35 [B]

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult frogs and capital letters for juvenile females).

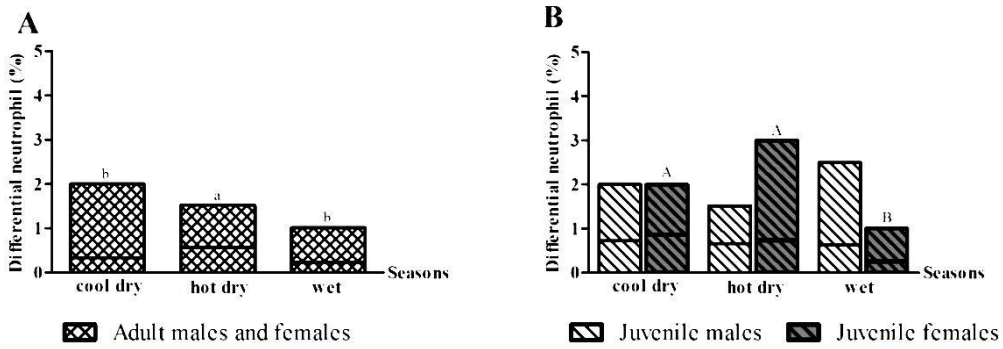

Figure 4-9 Differential neutrophil counts (mean and range) of **(A)** adult and **(B)** juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters (small letters for adult frogs and capital letters for juvenile females).

1.2.5.4 Differential basophil count

The values of differential basophil count of peripheral blood of *H. rugulosus* are summarized in Table 4-9 and Figure 4-10. In cool dry season, the mean value of differential basophil count of adult *H. rugulosus* was 0.30 ± 0.50 %. The mean value of differential basophil count of juvenile *H. rugulosus* was 0.54 ± 0.70 %. In hot dry season, the mean value of differential basophil count of adult *H. rugulosus* was 0.34 ± 0.50 %. The mean value of differential basophil count of juvenile *H. rugulosus* was 0.34 ± 0.50 %. In wet season, the mean value of differential basophil count of adult *H. rugulosus* was 0.37 ± 0.70 %. The mean value of differential basophil count of juvenile *H. rugulosus* was 0.57 ± 0.60 %.

After seasonal variation analysis (Table 4-9, Figure 4-10), the result showed that the mean values of differential basophil count of adult and juvenile *H. rugulosus* were not significantly different among seasons (one-way ANOVA, H = 0.67, df = 2, $P = 0.715$ in adult and one-way ANOVA, H = 2.68, df = 2, $P = 0.262$ in juvenile).

Table 4-9 Differential basophil counts (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Differential basophil counts (%)	
	Adult	Juvenile
	Males & Females (n=40)	Males & Females (n=40)
Cool dry	0.30 ± 0.50	0.54 ± 0.70
Hot dry	0.34 ± 0.50	0.34 ± 0.50
Wet	0.37 ± 0.70	0.57 ± 0.60

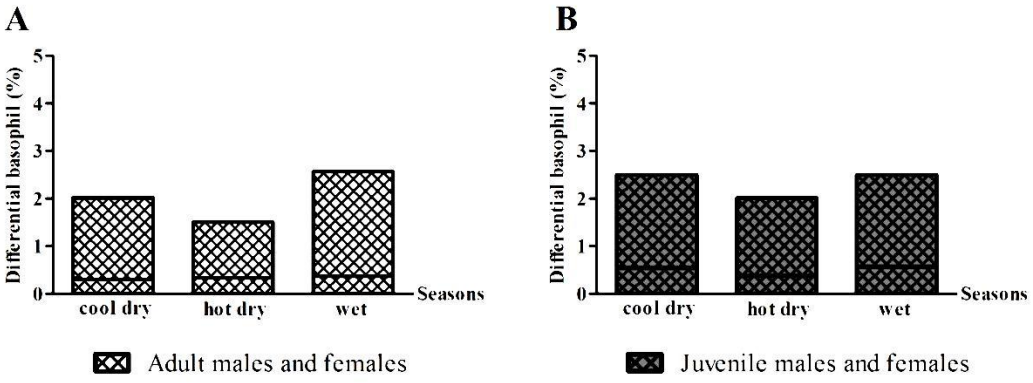

Figure 4-10 Differential basophil counts (mean and range) of **(A)** adult and **(B)** juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

1.2.5.5 Differential eosinophil count

The values of differential eosinophil count of peripheral blood of *H. rugulosus* are summarized in Table 4-10 and Figure 4-11. In cool dry season, the mean values of differential eosinophil count of adult *H. rugulosus* were 9.27 ± 3.37 and 5.81 ± 2.80 % in males and females, respectively. The mean values of differential eosinophil count of juvenile *H. rugulosus* were 7.26 ± 2.69 and 8.38 ± 3.11 % in males and females, respectively. In hot dry season, the mean values of differential eosinophil count of adult *H. rugulosus* were 7.59 ± 2.73 and 7.89 ± 3.61 % in males and females, respectively. The mean values of differential eosinophil count of juvenile *H. rugulosus* were 9.67 ± 3.03 and 9.73 ± 2.66 % in males and females, respectively. In wet season, the mean values of differential eosinophil count of adult *H. rugulosus* were 8.85 ± 4.59 and 7.75 ± 4.37 % in males and females, respectively. The mean values of differential eosinophil count of juvenile *H. rugulosus* were 12.07 ± 2.87 and 7.73 ± 2.12 % in males and females, respectively.

After seasonal variation analysis (Table 4-10, Figure 4-11), the result showed that the mean values of differential eosinophil count of adult *H. rugulosus* were not significantly different among all seasons. But the mean values of differential eosinophil count of juvenile males *H. rugulosus* were significantly different among all seasons (one-way ANOVA, $F = 14.10$, $df = 2$, $P < 0.001$).

Table 4-10 Differential eosinophil counts (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Differential eosinophil counts (%)			
	Adult		Juvenile	
	Males (n=20)	Females (n=20)	Males (n=20)	Females (n=20)
Cool dry	9.27 ± 3.37	5.81 ± 2.80	7.26 ± 2.69 [c]	8.38 ± 3.11
Hot dry	7.59 ± 2.73	7.89 ± 3.61	9.67 ± 3.03 [b]	9.73 ± 2.66
Wet	8.85 ± 4.59	7.75 ± 4.37	12.07 ± 2.87 [a]	7.73 ± 2.12

Remark: Significant difference between seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters.

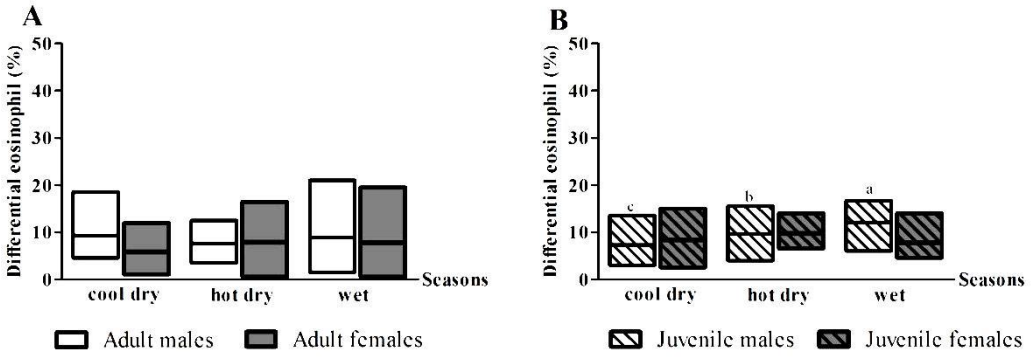

Figure 4-11 Differential eosinophil counts (mean and range) of **(A)** adult and **(B)** juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remark: Significant difference among seasons (one-way ANOVA, $p < 0.05$) is indicated by different superscript letters.

2. Normal reference values of the hematological parameters

The hematological normal reference values of farm raised *H. rugulosus* were determined separately between sexes (if the significant difference was presented), adult/juvenile and among seasons. All hematological parameters were defined for normal reference range values by minimum and maximum values (if $n \leq 40$) or mean ± 2SD (if $n > 40$). The ranges of the normal reference values of hematological parameters are showed in Table 4-11 to Table 4-20.

Table 4-11 Ranges of the normal reference values of erythrocyte count of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Normal reference values of erythrocyte count ($\times 10^6$ cells/µL)			
	Adult		Juvenile	
	Male	Female	Male	Female
Cool dry	0.32-0.85	0.45-0.89	0.32-1.00	0.49-1.59
Hot dry	0.73-1.18	0.40-0.72	1.03-1.35	0.37-1.04
Wet	0.61-1.97	1.37-1.69	1.54-2.28	0.82-1.32

Table 4-12 Ranges of the normal reference values of leukocyte count of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Normal reference values of leukocyte count ($\times 10^3$ cells/µL)	
	Adult	Juvenile
	Male & Female	Male & Female
Cool dry	1.85-8.25	2.00-4.38
Hot dry	2.10-10.83	1.85-5.60
Wet	2.48-11.50	3.75-8.10

Table 4-13 Ranges of the normal reference values of PCV of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Normal reference values of PCV (%)			
	Adult		Juvenile	
	Male	Female	Male	Female
Cool dry	28.00-45.50	17.50-47.50	30.00-53.00	29.50-49.00
Hot dry	36.50-56.00	29.00-46.50	34.50-51.00	25.00-43.50
Wet	30.50-55.50	26.00-48.00	37.50-50.00	31.50-46.50

Table 4-14 Ranges of the normal reference values of plasma total protein of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Normal reference values of plasma total protein (mg/mL)			
	Adult		Juvenile	
	Male	Female	Male	Female
Cool dry	45.51-84.94	60.78-116.25	30.84-81.69	23.50-131.87
Hot dry	29.91-75.68	26.52-73.12	27.95-62.29	30.68-74.34
Wet	26.19-81.55	33.65-83.33	48.97-91.97	48.17-83.57

Table 4-15 Ranges of the normal reference values of differential lymphocyte count of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Normal reference values lymphocyte count (%)			
	Adult		Juvenile	
	Male	Female	Male	Female
Cool dry	38.50-66.00	44.72-70.56	50.50-73.58	46.50-66.83
Hot dry	48.50-67.01	42.71-82.50	46.19-63.78	46.43-64.65
Wet	62.50-80.20	63.00-80.00	52.00-72.45	57.79-76.38

Table 4-16 Ranges of the normal reference values of differential monocyte count of adult and juvenile *H. rugulosus*, collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Normal reference values of differential monocyte count (%)		
	Adult		Juvenile
	Male	Female	Male & Female
Cool dry	26.26-54.00	26.40-53.00	21.24-43.50
Hot dry	26.40-43.50	10.61-43.50	25.50-41.84
Wet	10.66-34.50	10.50-30.81	11.62-37.50

Table 4-17 Ranges of the normal reference values of differential neutrophil count of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Normal reference values of differential neutrophil count (%)		
	Adult	Juvenile	
	Male & Female	Male	Female
Cool dry	0.00-2.00	0.00-2.00	0.00-2.00
Hot dry	0.00-1.50	0.00-1.52	0.00-1.52
Wet	0.00-1.02	0.00-1.52	0.00-0.50

Table 4-18 Ranges of the normal reference values of differential basophil count of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Normal reference values of differential basophil count (%)	
	Adult	Juvenile
	Male & Female	Male & Female
Cool dry	0.00-2.02	0.00-2.50
Hot dry	0.00-1.51	0.00-2.02
Wet	0.00-2.56	0.00-1.53

Table 4-19 Ranges of the normal reference values of differential eosinophil count of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Normal reference values of differential eosinophil count (%)			
	Adult		Juvenile	
	Male	Female	Male	Female
Cool dry	4.50-13.13	1.02-12.00	3.00-13.50	2.55-15.00
Hot dry	3.50-12.50	0.50-16.58	4.02-15.50	6.50-14.07
Wet	1.50-21.00	0.51-19.50	6.00-16.67	4.50-14.07

Discussion

This study is the first report of the hematological parameter values and the normal reference values of farm raised *H. rugulosus*. The hematological parameter values of some farm raised anurans were previously reported such as in African clawed frog (*Xenopus laevis*; Chang et al., 2015; Wilson et al., 2011), American bullfrog (*Lithobates catesbeianus*; Sombatboon, 2015; Teixeira et al., 2012) and wild Australian tree frogs (Australian green tree frogs, *Litoria caerulea* and white-lipped tree frog, *Litoria infrafrenata*) by Young et al. (2012).

The mean numbers of erythrocyte counts of *H. rugulosus* in all seasons were similar to those previously reported in *X. laevis* (Chang et al., 2015; Wilson et al., 2011), but higher than those of *L. catesbeianus* (Teixeira et al., 2012). The mean numbers of leukocyte counts in all seasons were higher than that previously reported in *X. laevis* raised in farms at Wisconsin and Florida, USA (Wilson et al., 2011) but lower than that reported in *X. laevis* raised in farm at California, USA (Chang et al., 2015). This indicates that even in the same species, the leukocyte counts are varied.

The PCV of these *H. rugulosus* showed a significant sex-related difference with a higher PCV value in adult males. The PCV value has been reported to indicate the erythrocyte mass in amphibians (Allender and Fry, 2008). However, the mean PCV value of *H. rugulosus* regardless of the gender (41.67 %) was higher than those reported in *L. catesbeianus* (Sombatboon, 2015; Teixeira et al., 2012) and *X. laevis* raised in farm at Wilconsin, USA (Wilson et al., 2011), but lower than those of *X. laevis* raised in farm at California, USA (Chang et al., 2015) and *X. laevis* raised in farm at Florida, USA (Wilson et al., 2011). The difference in PCV values among amphibians is believed to depend on differences in sex, season, habitat and natural history of each species (Allender and Fry, 2008; Campbell, 2015).

The plasma total protein is a quantitative measurement of the concentration of all proteins presenting in plasma. The major proteins are albumin and globulin, which contributes to 93 % of the plasma total protein. Many other proteins are also included in the measurement but contribute to not more than 5% of the total protein in serum (Salakij, 2005). The mean values of plasma total protein of adult and juvenile farm

raised *H. rugulosus* were higher than those previously reported in *X. laevis* (Chang et al., 2015; Wilson et al., 2011) and in *L. catesbeianus* (Sombatboon, 2015).

The differential count of leukocyte is one of the parameters that can be used to assess the risk of infection within the body (Bain et al., 2012). The values of differential leukocyte counts showed a wide variation depended on differences in sex, age and seasons (Romanova and Egorikhina, 2006; Young et al., 2012). From this study, the differential lymphocyte and neutrophil counts of farm raised *H. rugulosus* showed sex-related difference with higher values in adult and juvenile females in all seasons. But, the values of the differential monocyte and eosinophil counts showed sex-related difference with higher values in adult and juvenile males in all seasons. Very few researches have reported sex-related differences of these parameters previously in amphibians (Das and Mahapatra, 2012; Davis and Maerz, 2008).

From the result of differential leukocyte counts, agranulocytes were found to comprise a seven-fold higher proportion of the leukocytes than granulocytes (91% and 9% of all leukocytes, respectively). The most abundant leukocytes in fram raised *H. rugulosus* peripheral blood were lymphocytes (63% of all leukocytes), which is similar to the previous reports in other frog species (Arikan and Cicek, 2014; Cabagna et al., 2005; Cathers et al., 1997; Das and Mahapatra, 2012; Singh, 1977b).

The hematological normal reference values were reported in laboratory-reared and wild-caught, *X. laevis* (Chang et al., 2015; Wilson et al., 2011) and wild Australian tree frog, *L. caerulea* and *L. infrafrenata* (Young et al., 2012). In comparison, the reported hematological normal reference values of these frogs were different from those of *H. rugulosus* reported in our study, some of them without factor analysis, such as sex-related, age, seasonal and environmental differences. The previous report by Wilson et al. (2011) on *X. laevis* that caught from different farms (*X. laevis* caught from NASCO Farm, Fort Atkinson,Wisconsin and from *Xenopus* Express Farm, Brookville, Florida, USA) found that the normal reference values were different between 2 populations of *X. laevis*. This may be because of different factors of farm conditions. So, it should be noted that even in the same species, the normal reference values might be different depending on farm conditions. The differences in

hematological values upon sex, seasonal and environmental factors were also reported in other amphibian species (Arikan and Cicek, 2014; Cabagna et al., 2005; Das and Mahapatra, 2012; Davis et al., 2008; Gul et al., 2011; Singh, 1977a; Singh, 1977b; Sinha, 1983).

Conclusion

This study firstly presented the hematological parameters of farm raised *H. rugulosus* as well as its normal reference values. The hematological parameters of adult *H. rugulosus* that showed sex-related difference include erythrocyte count, PCV, plasma total protein, differential lymphocyte count, differential monocyte count and differential eosinophil count. The parameters of juvenile *H. rugulosus* that showed sex-related difference include PCV, Plasma total protein, differential lymphocyte count, differential monocyte count, differential neutrophil count and differential eosinophil count. After seasonal variation analyses we found that all parameters of adult *H. rugulosus* showed seasonal variation except differential basophil count and differential eosinophil count. Most of parameters of the juveniles also showed seasonal variation except differential basophil count. The normal reference values were therefore established and recommended to use separately between sexes, among age groups and among seasons (in case the parameter showed significant sex-related difference). The established normal reference values will be crucial basic data for further study in hematology of farm raised *H. rugulosus* in the future.

CHAPTER V
VALIDATION OF HEMATOLOGICAL NORMAL REFERENCE VALUES OF FARM RAISED RICE FIELD FROG *Hoplobatrachus rugulosus*

Introduction

Hematological parameters are primarily used to evaluate the health of animals and humans. In amphibians, several studies have described the general characteristics of the blood profile (Arikan and Cicek, 2014; Burgmeier et al., 2011; Cabagna et al., 2005; Das and Mahapatra, 2012; Shutler and Marcogliese, 2011; Singh, 1977a; Singh, 1977b; Zhelev et al., 2006), but there are many species that their normal reference values are still unknown or imprecise. However, the normal reference values have been reported in some amphibian species including African clawed frog (*Xenopus laevis*; Chang et al., 2015; Wilson et al., 2011), Australian green tree frog (*Litoria caerulea*) and white-lipped tree frog (*Litoria infrafrenata*; Young et al., 2012).

Hematological normal reference value is a set of value resulting from blood sample test of healthy animals and being used by a health professional to interpret a set of medical test results. It is a basis for a physician to interpret a set of test results for a particular domestic animal and human patient. The hematological test result that falls out of the range of normal reference values can indicate abnormality of the patient's health status and it is needed for further advanced investigation. Once the normal reference values were established in a species, they may need to be validated in order to assure that the values are an appropriate representative of normal parameter values in healthy animals. In this study, the normal reference values of farm raised *H. rugulosus* were established (Chapter IV) and needed to be validated. When the normal reference value was proven to be sensitive enough, it will be very useful for assessment of health status of this farm raised *H. rugulosus* in the future. So, the objective of this study is to validate the hematological normal reference values of farm raised *H. rugulosus*.

Materials and Methods

1. Frog collection

Eighty-six adult sick *H. rugulosus* (male = 40, female = 46) were selected in 3 seasons from the Huai Hong Khrai Royal Development Study Center (UTM 523200 2807200, zone line 47Q), Chiang Mai Province, northern Thailand. The sampling periods were February (cool dry season), May (hot dry season) and August (wet season) in 2014. The seasons were defined based on a climograph plotted between mean values of temperature and total rainfall (Walter et al., 1975). The sick frogs were observed for external morphology of disease symptoms. It was found that there were many kinds of disease symptoms in farm raised *H. rugulosus* including bacterial and viral infectious wounds, fungal infectious wounds, corneal edema or corneal opacity, blindness, hemorrhage of belly skin and leg, torticollis, edema, emaciation and enteritis. The disease symptom characteristics of sick frogs were summarized in Table 5-1 to 5-3 and Figure 5-1 to 5-2. The symptoms including redness or swelling, pus discharge and bad odor of the wound indicate that the diseases were caused by bacterial and viral infection (Berger et al., 1999; Densmore and Green, 2007; Johnson and Wellehan, 2005; Mauel et al., 2002). The visible colonies of mold (white patch) on the wound indicate that the diseases were caused by fungal infection (Megan and Richard, 2005; Romansic et al., 2009). The corneal edema or corneal opacity was characterized by the eyes over-protrude from eye socket. Some sick frogs showed damaged lens and abnormal characteristics of cornea and eye balls (Densmore and Green, 2007; Shilton et al., 2001). The blindness case could be characterized by loss of the eyeball from eye socket. The enteritis was caused by chronic inflammation of the intestine (Densmore and Green, 2007) which can be seen as the intestine's polyp protruding out of cloaca. The torticollis could be characterized by the frog's head twisting and turning to one side (Berger et al., 1999; Olson et al., 1992). The hemorrhage of belly skin could be characterized by numerous small red pimples or patches on the frog skin (Johnson and Wellehan, 2005). The edema could be characterized by abdominal swelling (Densmore and Green, 2007; Wolf et al., 1968). The emaciation was defined by an extremely weight loss and unnatural thinness due to a loss of subcutaneous fat (Densmore and Green, 2007).

Figure 5-1 Symptoms generally appear on the adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province. (**A**) Wounds on the snout and head (arrows). (**B**) Wounds on feet and toes (arrow). (**C**) Colonies of mold on the wound (arrows). (**D**) Hemorrhage of eye (arrow). (**E**) Hemorrhage of nictitating membrane (arrow). (**F**) Corneal opacity or corneal edema (arrow).

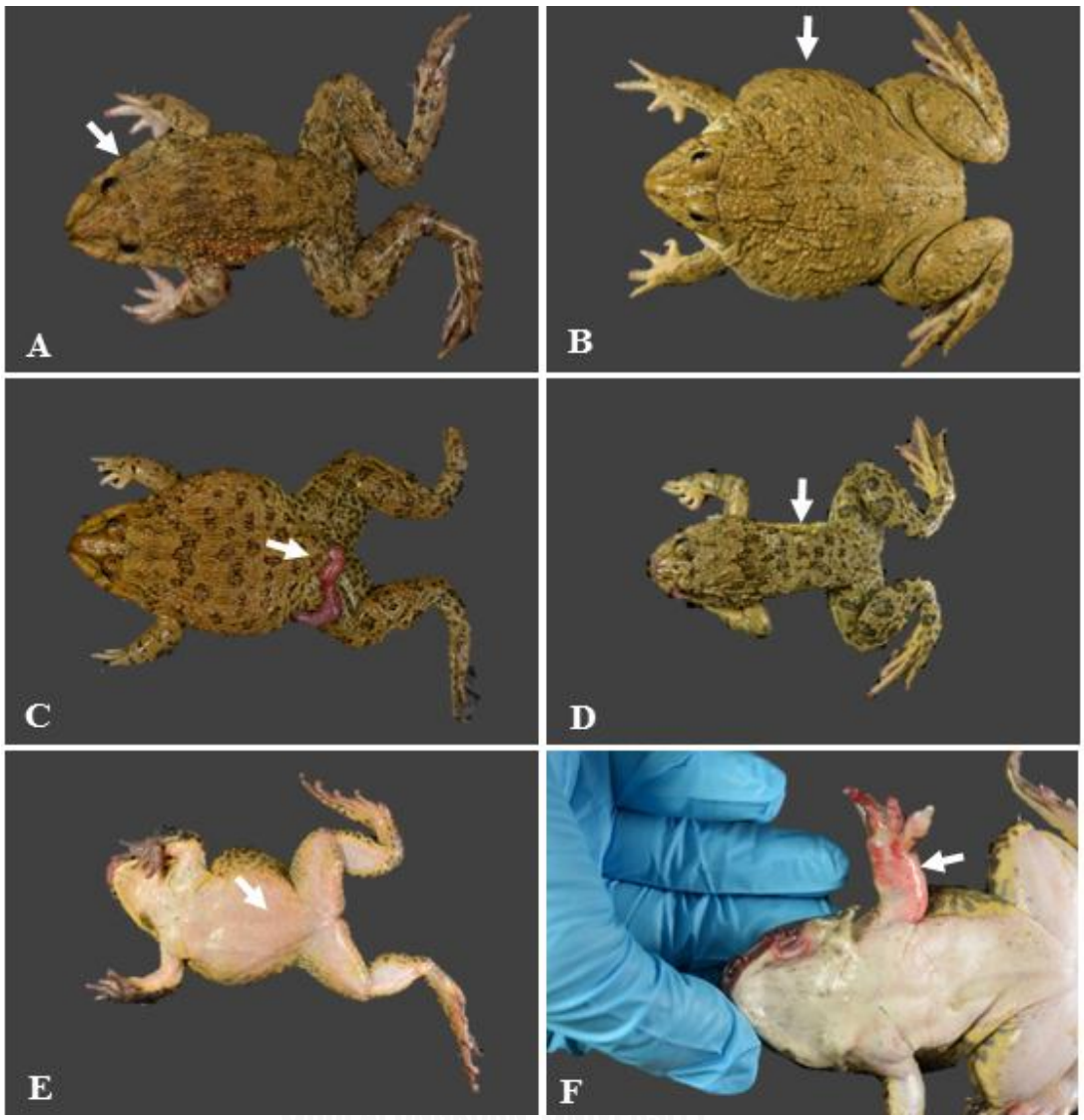

Figure 5-2 Symptoms generally appear on the adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province. (**A**) Torticollis (arrow). (**B**) Edema (arrow). (**C**) Enteritis (arrow). (**D**) Emaciation (arrow). (**E**) Hemorrhage of belly skin (arrow). (**F**) Hemorrhage of leg (arrow).

Table 5-1 Disease symptom characterization of adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in cool dry season 2014

Code	Sex	Disease symptoms														
		Bacterial and viral infectious wound			Fungal infectious wound			Corneal edema	Blindness	Hemorrhage			Torticollis	Edema	Emaciation	Enteritis
		Feet	Head	Skin	Feet	Head	Skin			eye	leg	body				
1HH01	M	✓	✓													
1HH02	M		✓													
1HH03	M	✓														
1HH04	M					✓		✓								
1HH05	M	✓	✓													
1HH06	M	✓	✓													
1HH07	M	✓														
1HH08	M								✓							
1HH09	M	✓	✓													
1HH10	M	✓					✓									
1HH11	M							✓								
1HH12	M	✓	✓													
1HH13	M		✓													
1HH14	M	✓						✓								
1HH15	M	✓							✓							
1HH16	M	✓														
1HH17	M	✓	✓													
1HH18	M		✓					✓								
1HH19	M	✓								✓						
1HH20	M	✓	✓													
1HH21	M	✓						✓								
1HH22	F		✓													
1HH23	F	✓	✓													
1HH24	F		✓													
1HH25	F		✓													
1HH26	F	✓								✓						
1HH27	F	✓	✓									✓				
1HH28	F	✓								✓						

Table 5-1 Disease symptom characterization of adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in cool dry season 2014 (continued)

Code	Sex	Disease symptoms														
		Bacterial and viral infectious wound			Fungal infectious wound			Corneal edema	Blindness	Hemorrhage			Torticollis	Edema	Emaciation	Enteritis
		Feet	Head	Skin	Feet	Head	Skin			eye	leg	body				
1HH29	F								✓							
1HH30	F		✓													
1HH31	F	✓	✓													
1HH32	F	✓	✓	✓									✓			
1HH33	F	✓	✓													✓
1HH34	F	✓												✓		
1HH35	F	✓	✓	✓												
1HH36	F	✓	✓											✓		
1HH37	F	✓	✓													
1HH38	F	✓	✓													
1HH39	F	✓										✓				
1HH40	F	✓	✓											✓		

Remarks: - Code 1HHXX are serial number of adult sick *H. rugulosus* in cool dry season.

- M and F are adult sick males and female, respectively.

Table 5-2 Disease symptom characterization of adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in hot dry season 2014

Code	Sex	Disease symptoms														
		Bacterial and viral infectious wound			Fungal infectious wound			Corneal edema	Blindness	Hemorrhage			Torticollis	Edema	Emaciation	Enteritis
		Feet	Head	Skin	Feet	Head	Skin			eye	leg	body				
2HH01	M	✓						✓								
2HH02	M	✓									✓					
2HH03	M							✓								
2HH04	M	✓	✓													
2HH05	M	✓											✓			
2HH06	F	✓						✓								
2HH07	F	✓			✓											
2HH08	F		✓						✓							
2HH09	F	✓														
2HH10	F	✓	✓													

Remarks: - Code 2HHXX are serial number of adult sick *H. rugulosus* in hot dry season.

- M and F are adult sick males and female, respectively

Table 5-3 Disease symptom characterization of adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in wet season 2014

Code	Sex	Disease symptoms														
		Bacterial and viral infectious wound			Fungal infectious wound			Corneal edema	Blindness	Hemorrhage			Torticollis	Edema	Emaciation	Enteritis
		Feet	Head	Skin	Feet	Head	Skin			eye	leg	body				
3HH01	M	✓														✓
3HH02	M	✓	✓	✓												
3HH03	M	✓	✓	✓	✓		✓									
3HH04	M			✓									✓			
3HH05	M				✓		✓									
3HH06	M										✓		✓			
3HH07	M										✓		✓			
3HH08	M	✓	✓													
3HH09	M	✓									✓					
3HH10	F	✓									✓					
3HH11	F		✓	✓												
3HH12	F	✓					✓									
3HH13	F												✓		✓	
3HH14	F	✓	✓													
3HH15	F			✓												
3HH16	F												✓	✓		
3HH17	F												✓			
3HH18	F										✓					
3HH19	F															✓
3HH20	F								✓							
3HH21	F												✓			
3HH22	F					✓	✓									
3HH23	F		✓	✓							✓					
3HH24	F												✓			
3HH25	F										✓	✓				
3HH26	F	✓		✓												
3HH27	F	✓		✓												
3HH28	F	✓		✓												

Table 5-3 Disease symptom characterization of adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in wet season 2014 (continued)

Code	Sex	Disease symptoms														
		Bacterial and viral infectious wound			Fungal infectious wound			Corneal edema	Blindness	Hemorrhage			Torticollis	Edema	Emaciation	Enteritis
		Feet	Head	Skin	Feet	Head	Skin			eye	leg	body				
3HH29	F	✓												✓		
3HH30	F		✓	✓							✓					
3HH31	F		✓	✓							✓					
3HH32	F	✓							✓							
3HH33	F			✓												
3HH34	F										✓		✓			
3HH35	F	✓		✓												
3HH36	F					✓										

Remarks: - Code 3HHXX are serial number of adult sick *H. rugulosus* in wet dry season.

- M and F are adult sick males and female, respectively

2. Blood sampling

Blood samples (0.5 mL per 100 g body weight) were collected from the sick frogs by cardiac puncture (Heatley and Johnson, 2009) under cold anesthesia using 25-gauge needle with heparinized tuberculin syringes and transferred to microcentrifuge tubes. The samples were stored in ice bucket during an hour of PCV, hemocytometer counting and blood smear processes.

3. Hematology

The PCV was determined after the blood sample had been transferred to a microcapillary tube and centrifuged at 8,700 ×g for 10 minutes (Salakij, 2005). Erythrocyte and leukocyte counts were determined manually using a hemocytometer (Tharp and Woodman, 2002) after the blood sample had been diluted with Natt and Herrick's solution (Natt and Herrick, 1951). Blood smears were prepared on glass slides immediately and fixed with absolute methanol. Giemsa staining (Bain et al., 2012) was used for the determination of hematological parameters using a light microscope at 400×magnification. On blood smear slide of each frog, a total of 200 leukocytes were counted for the differential leukocyte count. Plasma total protein were determined by Bradford assay (Bradford, 1976).

4. Validation of the normal reference values

All values of each hematological parameter of adult sick *H. rugulosus* were plotted on a graph of each corresponding hematological normal reference values of adult farm raised *H. rugulosus* (result from Chapter IV). The validation was done by the observation that the hematological values of adult sick frog fell inside or outside normal reference value range on the graph. The percentage of sick individual that has the value fell outside the range of normal reference value range was evaluated. The disease symptoms of those whose values fell outside the normal reference range were observed and analysed for parameter-specificity, if possible, for example the parameter that is sensitive for bacterial infection symptoms. If the normal reference values can discriminate more than 60 - 74 % of sick frogs from healthy frogs, it will be considered as a moderately sensitive reference values. If the normal reference

values can discriminate more than 75 % of sick frogs from healthy frogs, it will be considered as a highly sensitive reference values.

Results

1. Validation of erythrocyte count normal reference values

The validation of erythrocyte count normal reference values of farm raised *H. rugulosus* is summarized in Figure 5-3. In cool dry season, 2 of 3 (66.67 %) sick male frogs had the erythrocyte count values more than the normal reference values whereas 1 of 3 (33.33 %) sick female frogs had the erythrocyte count values less than the normal reference values. In hot dry season, 2 of 5 (40.00 %) sick males had the erythrocyte count values more than the normal reference values whereas 3 of 5 (60.00 %) sick females had the erythrocyte count values less than the normal reference values. In wet season, 1 of 6 (16.67 %) sick males had the erythrocyte count values less than the normal reference value whereas 6 of 8 (75.00 %) sick females had the erythrocyte count values less than the normal reference values.

2. Validation of leukocyte count normal reference values

The validation of leukocyte count normal reference values of farm raised *H. rugulosus* is summarized in Figure 5-4. In cool dry season, none of sick frogs had the leukocyte count values falling outside the range of the normal reference values. In hot dry season, 3 of 10 (30.00 %) sick frogs had the leukocyte count values less than the normal reference values. In wet season, none of sick frogs had the leukocyte count values falling outside the range of the normal reference values.

3. Validation of packed cell volume normal reference values

The validation of PCV normal reference values of farm raised *H. rugulosus* is summarized in Figure 5-5. In cool dry season, 4 of 21 (19.05 %) sick males had the PCV values less than the normal reference values whereas 1 of 19 (5.26 %) sick females had the PCV values more than the normal reference values. In hot dry season, 2 of 5 (40.00 %) sick males had the PCV values less than the normal reference values whereas 3 of 5 (60.00 %) sick females had the PCV values less than the normal reference values. In wet season, 6 of 9 (66.67 %) sick males had the PCV values less

than the normal reference values whereas 4 of 27 (14.81 %) sick females had the PCV values less than the normal reference values. But, 1 of 27 (3.70 %) sick females had the PCV values more than the normal reference values.

4. Validation of plasma total protein normal reference values

The validation of plasma total protein normal reference values of farm raised *H. rugulosus* is summarized in Figure 5-6. In cool dry season, 17 of 21 (80.95 %) sick male frogs had the plasma total protein values less than the normal reference values whereas all of sick female frogs (100 %) had the plasma total protein values less than the normal reference values. In hot dry season, none of sick males and sick females had the plasma total protein values falling outside the range of the normal reference values. In wet season, 1 of 9 (11.11 %) sick males had the plasma total protein values less than the normal reference values whereas 4 of 27 (14.81 %) sick females had the plasma total protein values less than the normal reference values.

5. Validation of differential leukocyte count normal reference values

5.1. Validation of differential lymphocyte count normal reference values

The validation of differential lymphocyte count normal reference values of farm raised *H. rugulosus* is summarized in Figure 5-7. In cool dry season, 1 of 21 (4.76 %) sick male frogs had the differential lymphocyte count values more than the normal reference values whereas 3 of 19 (16 %) sick female frogs had the differential lymphocyte count values less than the normal reference values. In hot dry season, 3 of 5 (60.00 %) sick males had the differential lymphocyte count values less than the normal reference values whereas none of sick females had the differential lymphocyte count values falling outside the range of the normal reference values. In wet season, 8 of 9 (89 %) sick males had the differential lymphocyte count values less than the normal reference values whereas 24 of 27 (88.89 %) sick females had the differential lymphocyte count values less than the normal reference values.

5.2. Validation of differential monocyte count normal reference values

The validation of differential monocyte count normal reference values of farm raised *H. rugulosus* is summarized in Figure 5-8. In cool dry season, none of sick

male and female frogs had the differential monocyte count values falling outside the range of the normal reference values. In hot dry season, 1 of 5 (20.00 %) sick males had the differential monocyte count values less than the normal reference values whereas 1 of 5 (20.00 %) sick females had the differential monocyte count values more than the normal reference values. In wet season, 4 of 9 (44.44 %) sick males had the differential monocyte count values more than the normal reference values whereas 20 of 27 (74.07 %) sick females had the differential monocyte count values more than the normal reference values.

5.3. Validation of differential neutrophil count normal reference values

The validation of differential neutrophil count normal reference values of farm raised *H. rugulosus* is summarized in Figure 5-9. In cool dry season, 4 of 40 (10.00 %) sick frogs had the differential neutrophil count values more than the normal reference values. In hot dry season, 2 of 10 (20.00 %) sick frogs had the differential neutrophil count values more than the normal reference values. In wet season, 9 of 36 (25.00 %) sick frogs had the differential neutrophil count values more than the normal reference values.

5.4. Validation of differential basophil count normal reference values

The validation of differential basophil count normal reference values of farm raised *H. rugulosus* is summarized in Figure 5-10. In cool dry season, 4 of 40 (10.00 %) sick frogs had the differential basophil count values more than the normal reference values. In hot dry season, 1 of 10 (10.00 %) sick frogs had the differential basophil count values more than the normal reference values. In wet season, 6 of 36 (16.67 %) sick frogs had the differential basophil count values more than the normal reference values.

5.5. Validation of differential eosinophil count normal reference values

The validation of differential eosinophil count normal reference values of farm raised *H. rugulosus* is summarized in Figure 5-11. In cool dry season, 4 of 21 (19.05 %) sick male frogs had the differential eosinophil count values less than the normal reference values whereas 2 of 21 (10 %) sick males had the differential eosinophil count values more than the normal reference values. In females, 8 of 19 (42.11 %)

sick frogs had the differential eosinophil count values more than the normal reference values. In hot dry season, 2 of 5 (40.00 %) sick males had the differential eosinophil count values less than the normal reference values whereas 1 of 5 (20.00 %) sicke females had the differential eosinophil count values more than the normal reference values. In wet season, none of sick males had the differential eosinophil count values falling outside the range of the normal reference values whereas 1 of 27 (3.70 %) sick females had the differential eosinophil count values more than the normal reference values. But, 3 of 27 (11.11 %) sick females had the differential eosinophil count values less than the normal reference values.

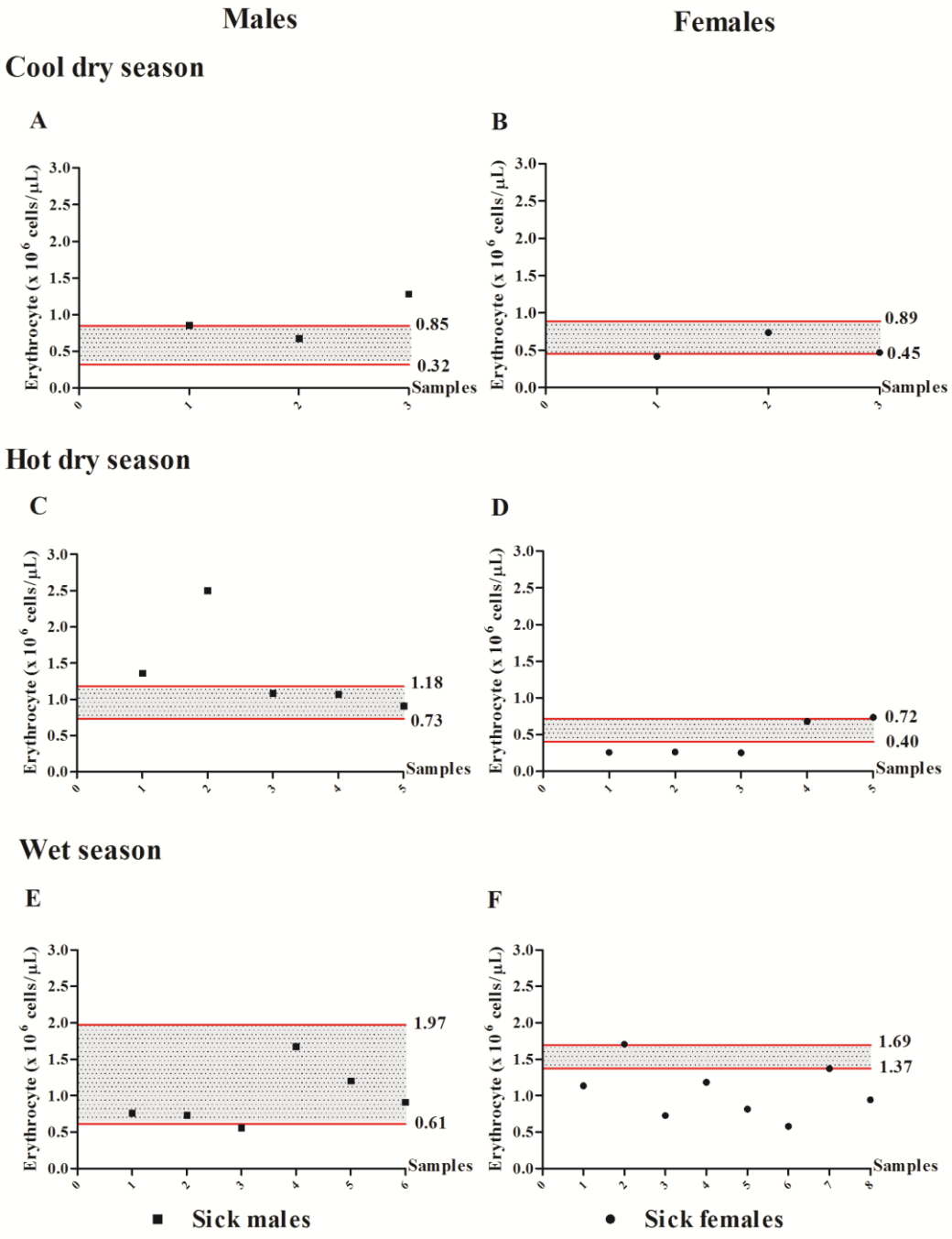

Figure 5-3 Validation plot of erythrocyte counts of sick farm raised *H. rugulosus* against the normal reference values from healthy male and female farm raised *H. rugulosus* in (**A-B**) cool dry season, (**C-D**) hot dry season and (**E-F**) wet season.

Remark: The highlight areas are the range of normal reference values.

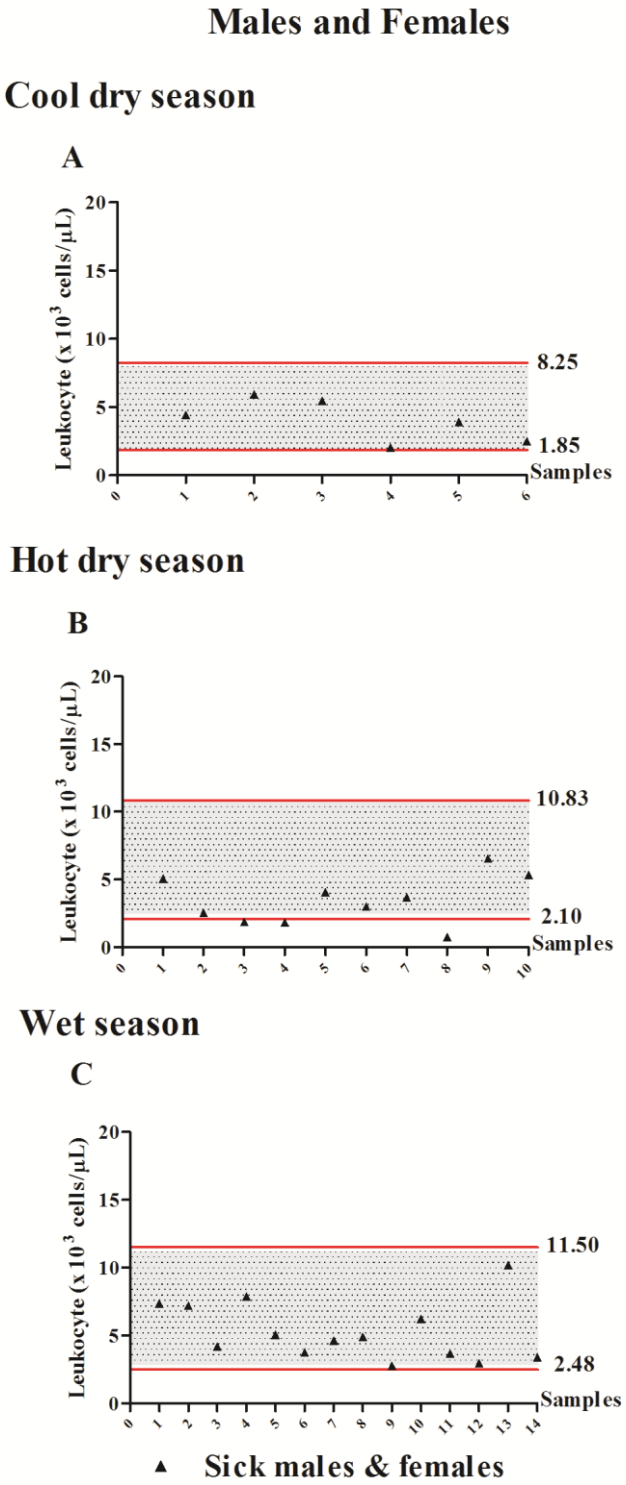

Figure 5-4 Validation plot of leukocyte counts of sick farm raised *H. rugulosus* against the normal reference values from healthy male and female farm raised *H. rugulosus* in (**A**) cool dry season, (**B**) hot dry season and (**C**) wet season.

Remark: The highlight areas are the range of normal reference values.

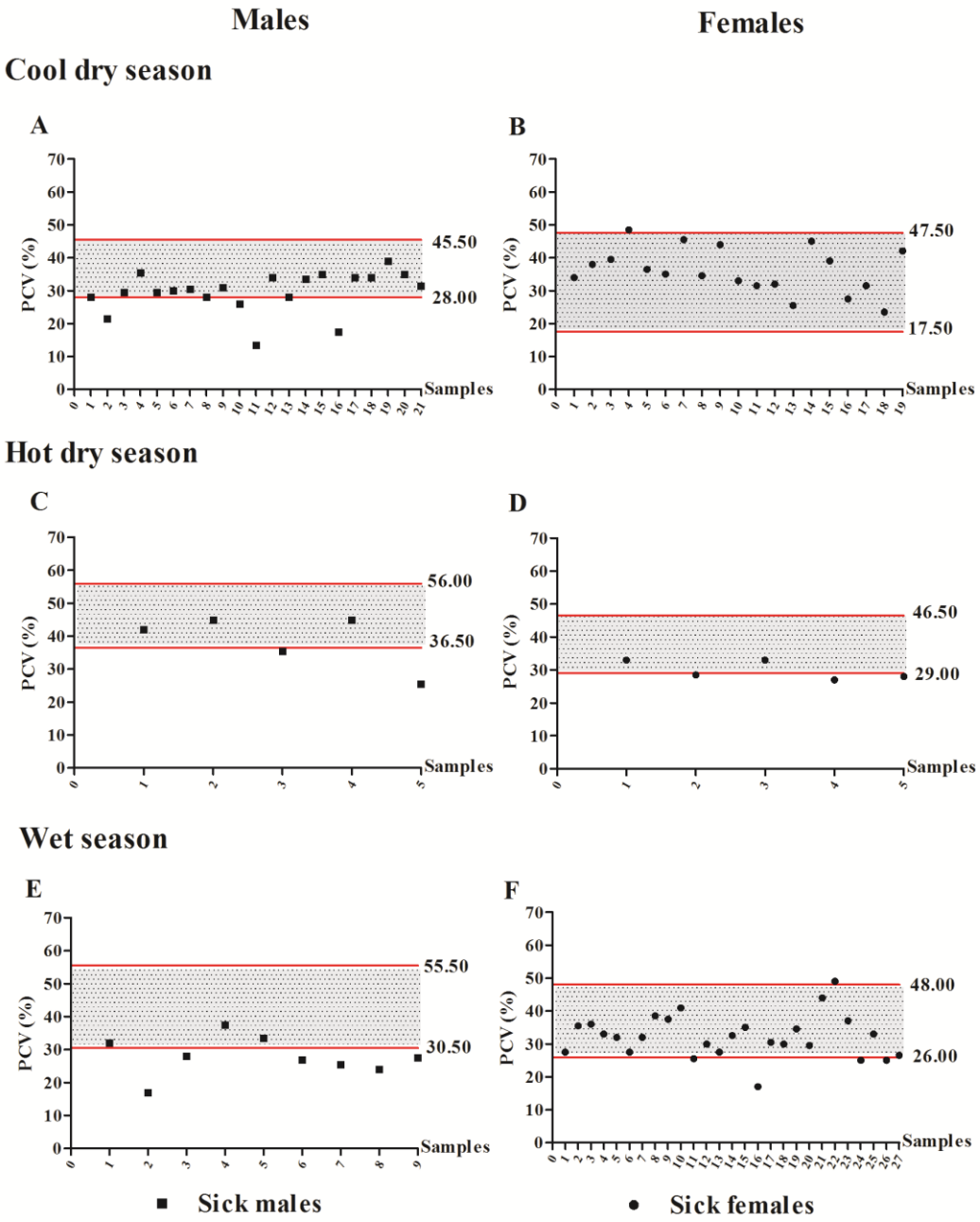

Figure 5-5 Validation plot of PCV of sick farm raised *H. rugulosus* against the normal reference values from healthy male and female farm raised *H. rugulosus* in (**A-B**) cool dry season, (**C-D**) hot dry season and (**E-F**) wet season.

Remark: The highlight areas are the range of normal reference values.

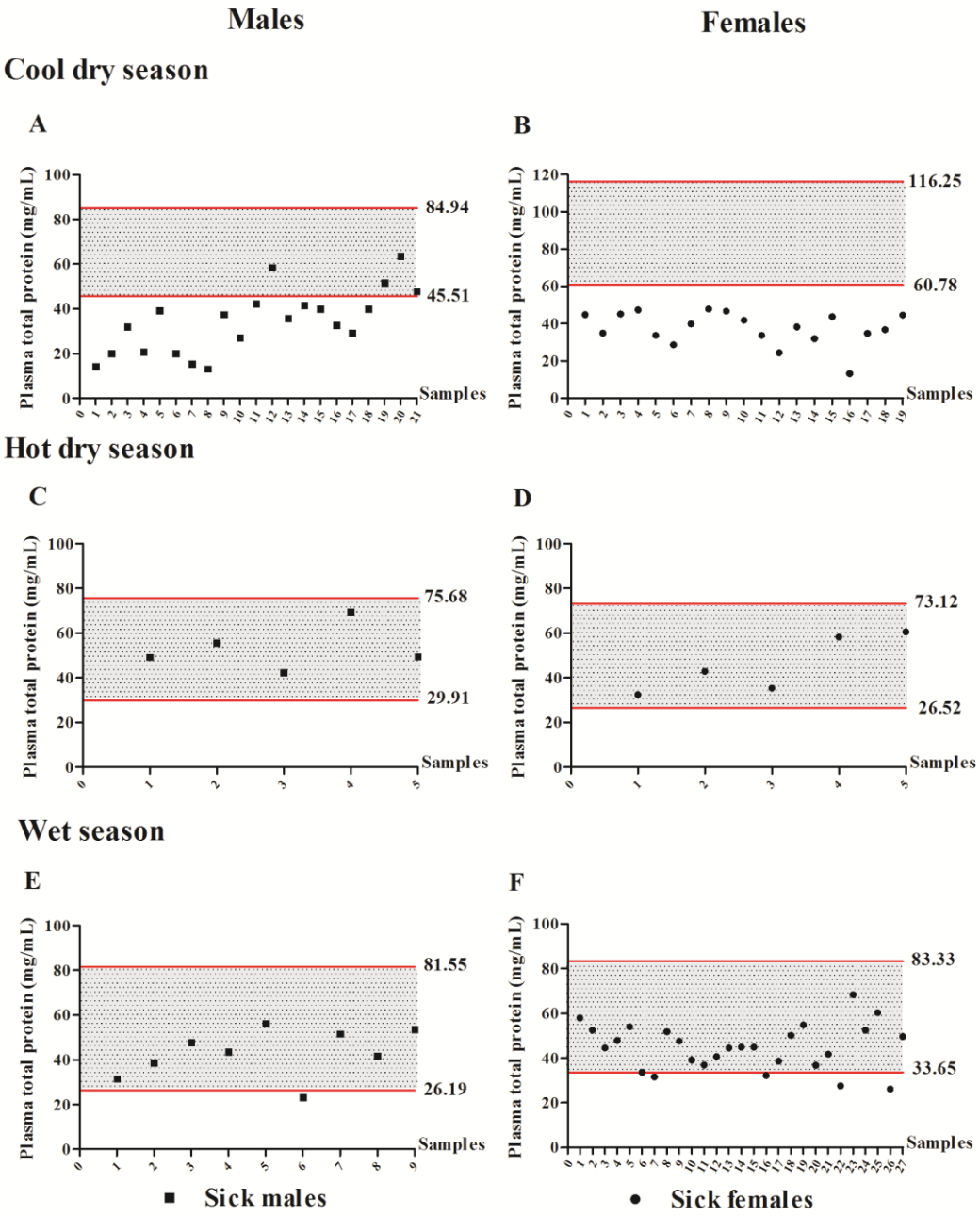

Figure 5-6 Validation plot of plasma total protein of sick farm raised *H. rugulosus* against the normal reference values from healthy male and female farm raised *H. rugulosus* in (**A**-**B**) cool dry season, (**C**-**D**) hot dry season and (**E**-**F**) wet season.

Remark: The highlight areas are the range of normal reference values.

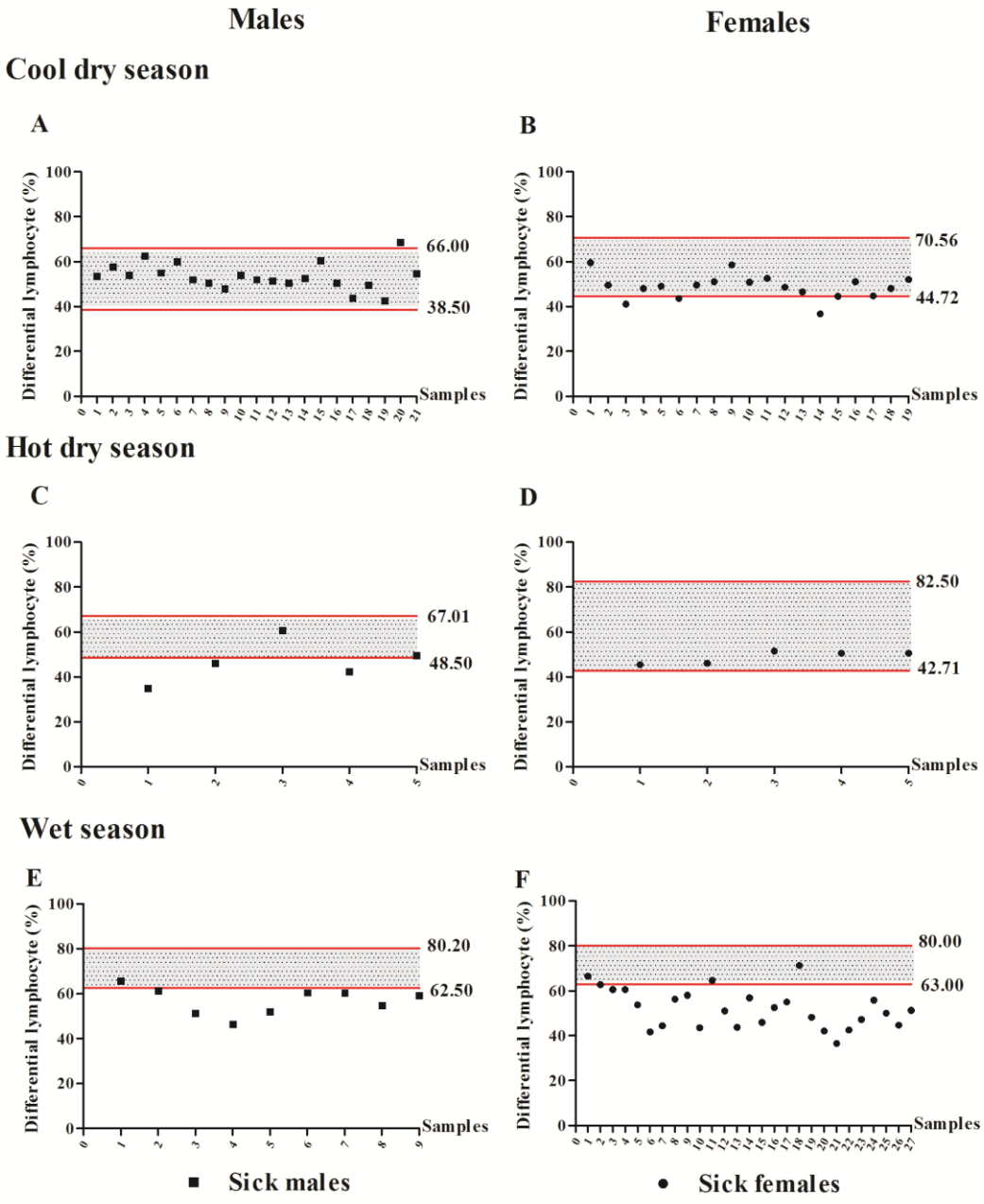

Figure 5-7 Validation plot of differential lymphocyte counts of sick farm raised *H. rugulosus* against the normal reference values from healthy male and female farm raised *H. rugulosus* in (**A**-**B**) cool dry season, (**C**-**D**) hot dry season and (**E**-**F**) wet season.

Remark: The highlight areas are the range of normal reference values.

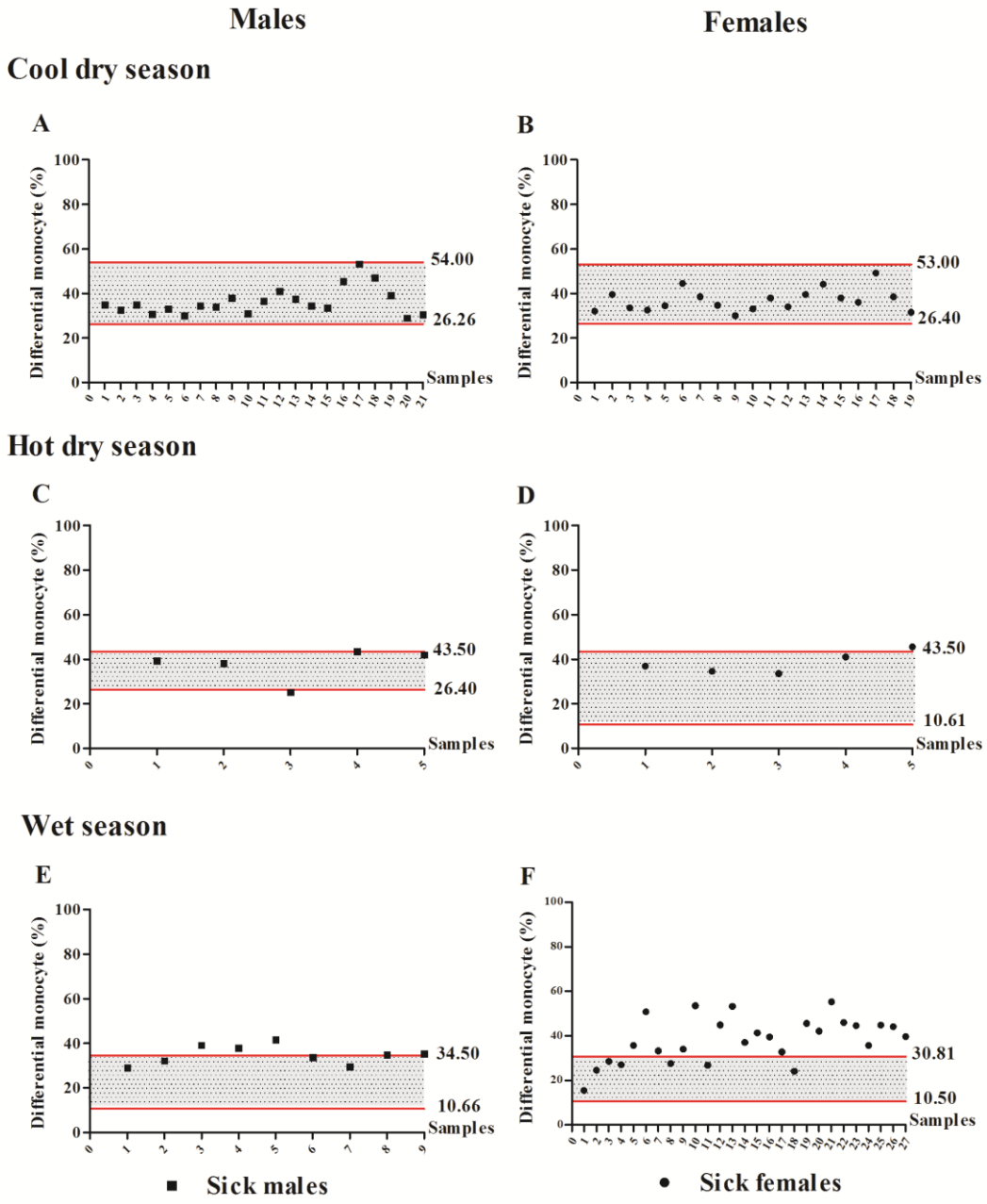

Figure 5-8 Validation plot of differential monocyte counts of sick farm raised *H. rugulosus* against the normal reference values from healthy male and female farm raised *H. rugulosus* in (**A-B**) cool dry season, (**C-D**) hot dry season and (**E-F**) wet season.

Remark: The highlight areas are the range of normal reference values.

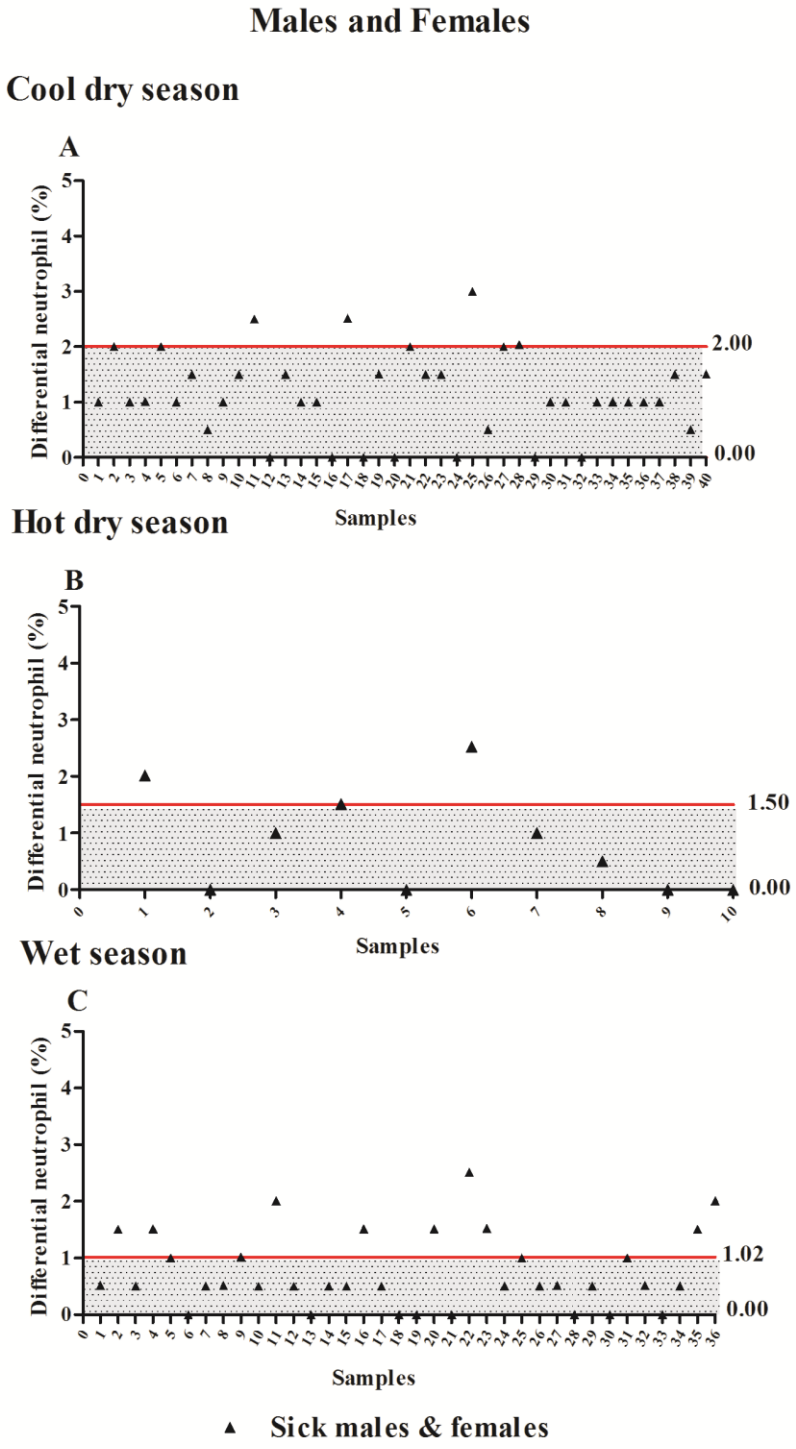

Figure 5-9 Validation plot of differential neutrophil counts of sick farm raised *H. rugulosus* against the normal reference values from healthy male and female farm raised *H. rugulosus* in (**A**) cool dry season, (**B**) hot dry season and (**C**) wet season.

Remark: The highlight areas are the range of normal reference values.

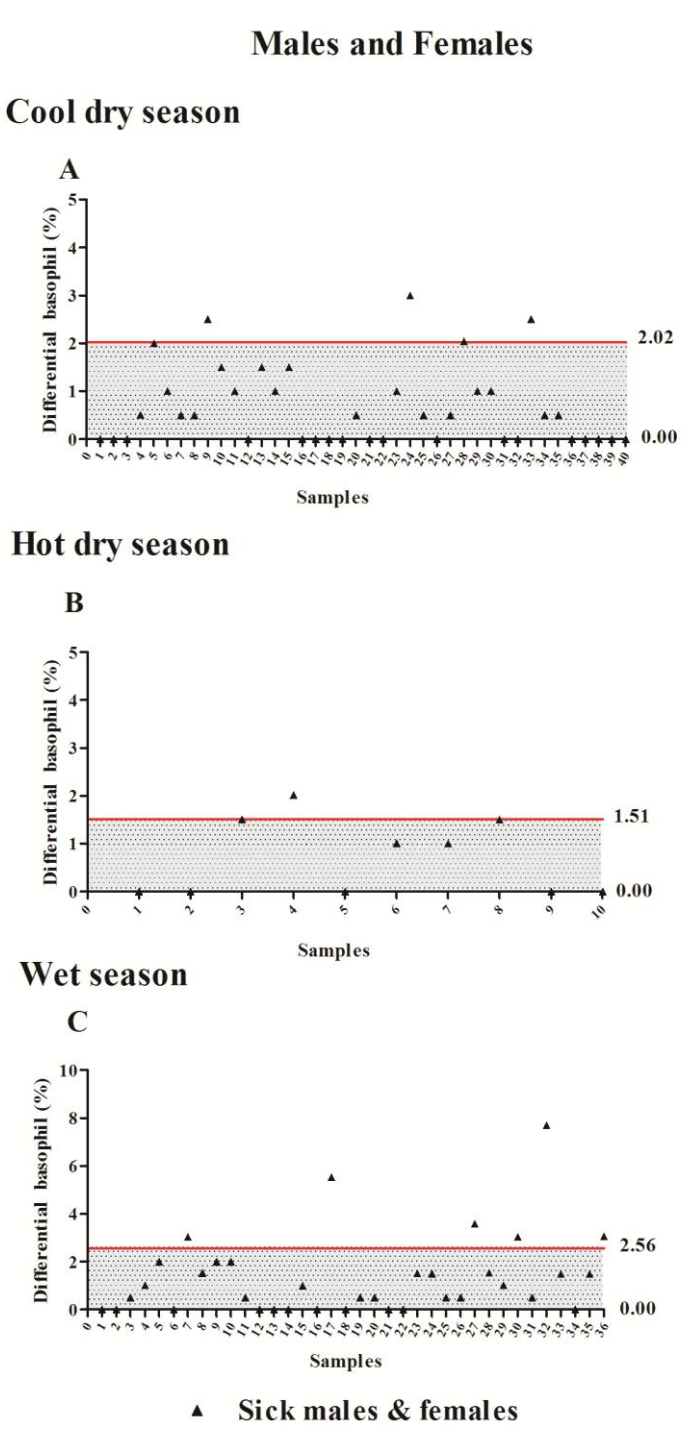

Figure 5-10 Validation plot of differential basophil counts of sick farm raised *H. rugulosus* against the normal reference values from healthy male and female farm raised *H. rugulosus* in (**A**) cool dry season, (**B**) hot dry season and (**C**) wet season.

Remark: The highlight areas are the range of normal reference values.

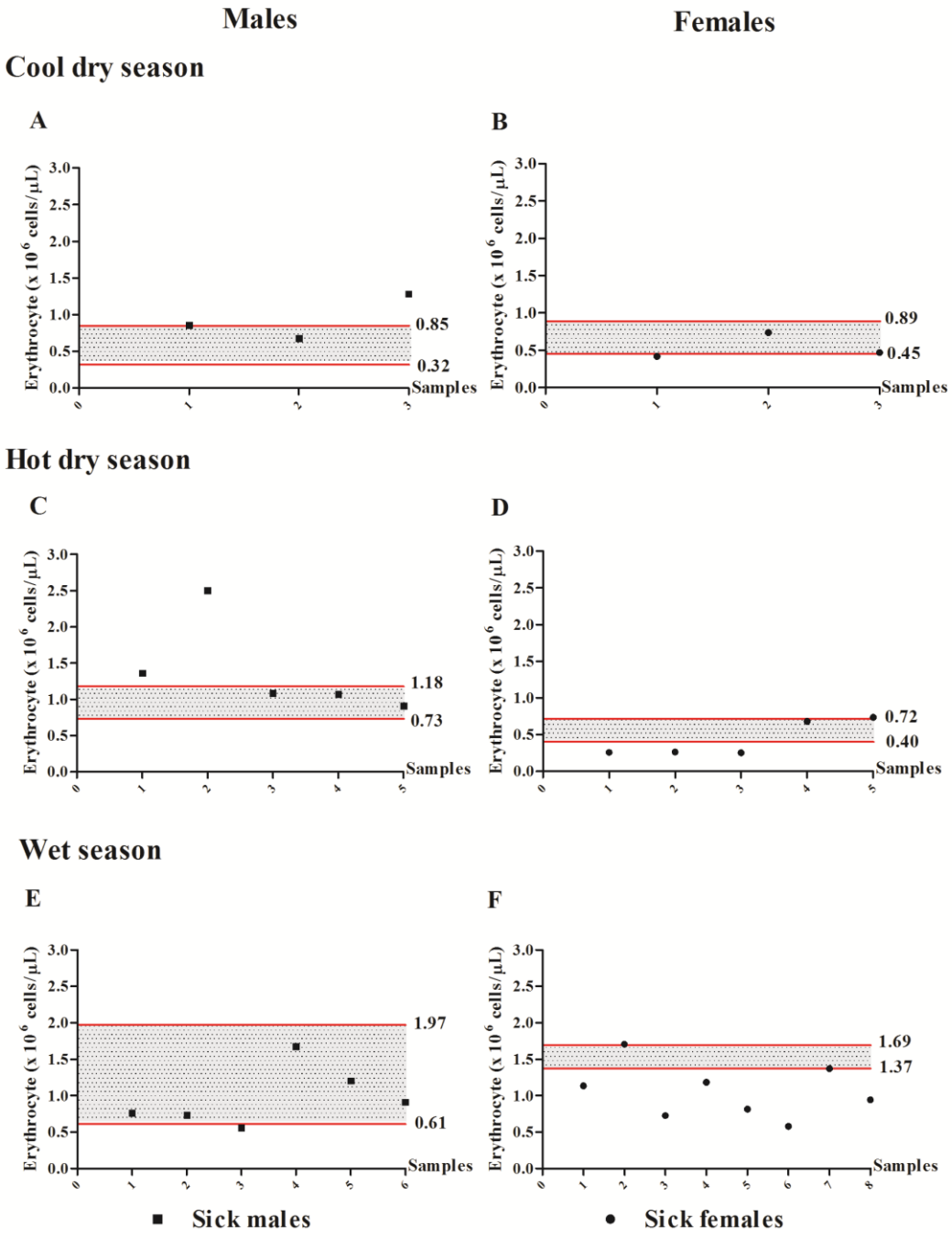

Figure 5-11 Validation plot of differential eosinophil counts of sick farm raised *H. rugulosus* against the normal reference values from healthy male and female farm raised *H. rugulosus* in (**A-B**) cool dry season, (**C-D**) hot dry season and (**E-F**) wet season.

Remark: The highlight areas are the range of normal reference values.

Discussion

The data of hematological normal reference values have been reported in some amphibian species (Chang et al., 2015; Wilson et al., 2011; Young et al., 2012). However, these values have not been reported about the usage of the hematological normal reference values after being established. This study is the first report of the validation of hematological normal reference values of farm raised *H. rugulosus*.

After validation, we found that some normal reference values of farm raised *H. rugulosus* that being established from this study could be used or have a potential to be develop to use in *H. rugulosus* farm system. Those with moderate sensitivity were the values that can discriminate than 60-74 % of sick frogs including erythrocyte count (male − cool dry season), differential lymphocyte count (male − hot dry season), erythrocyte count (female − hot dry season), PCV (female − hot dry season and male − wet season) and differential monocyte count (female − wet season). The normal reference values with high sensitivity were the values that can discriminate more than 75 % of sick frogs including erythrocyte count (female − wet season), plasma total protein (male and female − cool dry season) and differential lymphocyte count (male and female − wet season).

After the observation of symptoms that can be sensitively discriminated by the erythrocyte count, the major symptoms include bacterial and viral infection wounds on head and torticollis. These symptoms were caused by bacterial and viral infection. This indicates that the normal reference values of erythrocyte count may be used to monitor bacterial and viral infection in the farm raised female *H. rugulosus* in wet season.

The major symptoms that can be sensitively discriminated by the plasma total protein are bacterial and viral infection wounds on head. These symptoms were caused by bacterial and viral infection. This indicates that the normal reference value of plasma total protein may be used to monitor bacterial and viral infection in the farm raised *H. rugulosus*, both males and females, in cool dry season. This value was found very sensitive from the result of this study. Monitoring of plasma protein level

was also proven to be a major technique to evaluate the health status in farmed rodents (Zaias et al., 2009).

The major symptoms that can be sensitively discriminated by the differential lymphocyte count include bacterial and viral infection wounds on head, hemorrhage of leg and torticollis. These symptoms were caused by bacterial and viral infection. This indicates that the normal reference values of differential lymphocyte count may be used to monitor bacterial and viral infection in the farm raised *H. rugulosus*, both males and females, in wet season. The differential lymphocyte counts were found lower than the normal reference values in sick frogs (89% of both males and females). Even though the relationship between stress and the number of lymphocytes in amphibians is still unclear (Young et al., 2012), the results from our study showed that bacterial and viral infections in wet season associated with the decrease of lymphocyte in farm raised *H. rugulosus.* Chronic stress like infections diseases was proven to induce reduction and apoptosis of lymphocyte in mammals (Yin et al., 2000).

Conclusion

The normal reference values of hematological parameter that established in this study and validated to be sensitively used to monitor the health status of farm raised *H. rugulosus* including erythrocyte count (female − wet season), plasma total protein (male and female − cool dry season) and differential lymphocyte count (male and female − wet season). These normal reference values are recommended to use in the evaluation of health status of adult farm raised *H. rugulosus* to reduce the risk of disease spreading in the farm. The application for using of hematological values as health indicator should be developed for frog farm management in the future.

CHAPTER VI
GENERAL CONCLUSION

Blood cell morphology and hematological parameters are important for evaluating and monitoring health status of animals. In this study, hematology of the rice field frog *H. rugulosus* living in nature and farm in Thailand were examined. Wild adult *H. rugulosus* were collected from organic rice fields with no history of pesticide usage for more than 10 years in Wiang Sa District, Nan Province, northern Thailand. Farm raised *H. rugulosus* was obtained from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province, northern of Thailand.

The first part of this study was carried out to investigate blood cell morphology and determine the hematological parameters of wild caught *H. rugulosus*. Samples of frog were collected during the rainy season (July to August 2014). The results showed that, based on their morphological characteristics, cells in the peripheral blood of *H. rugulosus* were classified into mature and immature erythrocytes, leukocytes (lymphocytes, monocytes, neutrophils, basophils and eosinophils) and thrombocytes. The presence of immature erythrocytes, even though frequently reported in vertebrates, is interesting since an increase in their circulating level can indicate a disturbance of the erythron. Thus, the data obtained in this study will be useful in assessing abnormality in the erythron of *H. rugulosus* in the future. Moreover, these results revealed that the packed cell volume (PCV) and the differential lymphocyte and neutrophil counts of male frogs were significantly higher than those of the female frogs. However, the size of each blood cell type was not significantly different between sexes of *H. rugulosus*. The hematological parameters presented in this study are the first report for *H. rugulosus* and so represent the crucial baseline data for wild *H. rugulosus* in Thailand that can be expanded to use for monitoring the health status of this anuran in the future.

The second part of this study was carried out to determine hematological parameters of farm raised *H. rugulosus*. The farm raised adult and juvenile *H. rugulosus* with healthy external morphology were collected from the study site. The

sampling periods included cool dry season (February 2014), hot dry season (May 2014) and wet season (August 2014). The hematological parameters of adult *H. rugulosus* that showed sex-related difference included erythrocyte count, PCV, plasma total protein, differential lymphocyte count, differential monocyte count and differential eosinophil count. The parameters of juvenile *H. rugulosus* that showed sex-related difference included PCV, plasma total protein, differential lymphocyte count, differential monocyte count, differential neutrophil count and differential eosinophil count. After seasonal variation analyses, it was found that all parameters of adult *H. rugulosus* showed seasonal variation except differential basophil count and differential eosinophil count. Most parameters of the juveniles also showed seasonal variation except differential basophil count. These hematological parameters will be crucial basic data for further study in hematology of farm raised *H. rugulosus* in the future.

The final part of this study was carried out to determine hematological normal reference values and validation of these normal reference values of farm raised adult and juvenile *H. rugulosus*. In this study, the hematological normal reference values of adult and juvenile frogs in 3 seasons (cool dry, hot dry and wet seasons) are shown in Table 6-1 to 6-3. The normal reference values were therefore established and recommended to use separately between sexes, among age groups and among seasons (in case the parameter showed significant sex-related difference). For validation, the farm raised adult *H. rugulosus* with unhealthy external morphology were collected from the study site, in the same sampling periods as the healthy frogs including cool dry season (February 2014), hot dry season (May 2014) and wet season (August 2014). Values of each hematological parameter of the sick frogs were plotted on a graph of normal reference range of adult *H. rugulosus*. The normal values of hematological parameter that validated to be sensitive for monitoring the health status of farm raised *H. rugulosus* included erythrocyte count (female − wet season), plasma total protein (male and female − cool dry season) and differential lymphocyte count (male and female − wet season). These normal reference values are recommended to use in the evaluation of health status of adult farm raised *H. rugulosus* to reduce the

risk of disease spreading in the farm. The application of hematological evaluation for the frogs should be developed for frog farm management in the future.

Recommendations

- Since the normal reference values of a frog species may depend on many environmental factors including farm condition, it is recommended that the normal reference values shown in this study would be best applicable for the farm with the same or similar raising condition with the Huai Hong Khrai Royal Development Study Center.
- The normal reference values of hematological parameters that were validated to be highly sensitive parameters for monitoring the health status of farm raised *H. rugulosus* are differential lymphocyte count and plasma total protein. It is recommended that each farm should establish its own reference values of differential lymphocyte count and plasma total protein.

Table 6-1 Range of the hematological normal reference values of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in cool dry season

Parameters	Adult		Juvenile	
	Males	Females	Males	Females
Erythrocyte (× 10^6 cells/µL)	0.32-0.85	0.45-0.89	0.32-1.00	0.49-1.59
Leukocyte (× 10^3cells/µL)	1.85-8.25		2.00-4.38	
PCV (%)	28.00-45.50	17.50-47.50	30.00-53.00	29.50-49.00
Plasma total protein (mg/mL)	45.51-84.94	60.78-116.25	30.84-81.69	23.50-131.87
Differential lymphocyte counts (%)	38.50-66.00	44.72-70.56	50.50-73.58	46.50-66.83
Differential monocyte counts (%)	26.26-54.00	26.40-53.00	21.24-43.50	
Differential neutrophil counts (%)	0.00-2.00		0.00-2.00	0.00-2.00
Differential basophil counts (%)	0.00-2.02		0.00-2.50	
Differential eosinophil counts (%)	4.50-13.13	1.02-12.0	3.00-13.50	2.55-15.00

Table 6-2 Range of the hematological normal reference values of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in hot dry season

Parameters	Adult		Juvenile	
	Males	Females	Males	Females
Erythrocyte (× 10^6 cells/µL)	0.73-1.18	0.40-0.72	1.03-1.35	0.37-1.04
Leukocyte (× 10^3cells/µL)	2.10-10.83		1.85-5.60	
PCV (%)	36.50-56.00	29.00-46.50	34.50-51.00	25.00-43.50
Plasma total protein (mg/mL)	45.51-84.94	60.78-116.25	27.95-62.29	30.68-74.34
Differential lymphocyte counts (%)	48.50-67.01	42.71-82.50	46.19-63.78	46.43-64.65
Differential monocyte counts (%)	26.40-43.50	10.61-43.50	25.50-41.84	
Differential neutrophil counts (%)	0.00-1.50		0.00-1.52	0.00-1.52
Differential basophil counts (%)	0.00-1.51		0.00-2.02	
Differential eosinophil counts (%)	3.50-12.50	0.50-16.58	4.02-15.50	6.50-14.07

Table 6-3 Range of the hematological normal reference values of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in wet season

Parameters	Adult		Juvenile	
	Males	Females	Males	Females
Erythrocyte (× 10^6 cells/µL)	0.61-1.97	1.37-1.69	1.54-2.28	0.82-1.32
Leukocyte (× 10^3cells/µL)	2.48-11.50		3.75-8.10	
PCV (%)	30.50-55.50	26.00-48.00	37.50-50.00	31.50-46.50
Plasma total protein (mg/mL)	26.19-81.55	33.65-83.33	48.79-88.18	49.84-86.54
Differential lymphocyte counts (%)	62.50-80.20	63.00-80.00	52.00-72.45	57.79-76.38
Differential monocyte counts (%)	10.66-34.50	10.50-30.81	11.62-37.50	
Differential neutrophil counts (%)	0.00-1.02		0.00-1.52	0.00-0.50
Differential basophil counts (%)	0.00-2.56		0.00-1.53	
Differential eosinophil counts (%)	1.50-21.00	0.51-19.50	6.00-16.67	4.50-14.07

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APPENDIX A

Blood Sampling & Morphological Data

A-I Calculation of blood sample volume

This study followed the blood sampling guidelines for amphibian by Heatley and Johnson (2009). Normally, the minimal total blood volume of aquatic amphibian such as *H. rugulosus* was calculated by the following formula:

Minimal total blood volume (TBV) = Body weight (BW; g) × 0.1

Minimal safe blood draw for frog was 5% to 10 % of TBV. So, the minimal safe blood draw for *H. rugulosus* was calculated by the following formula:

Minimal safe blood draw (SBD) = 5% of TBV

= 0.05 × TBV

Example

For a 150 g the frog,

SBD = 0.05 × (150 × 0.1) = 0.75 mL

A-II Morphological data of farm raised and wild caught *H. rugulosus*

Morphological data of farm raised *H. rugulosus* were summarized in Table A-1 to A-9 and morphological data of wild caught *H. rugulosus* was summarized in Table A-10.

Table A-1 Morphological data of farm raised adult *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in cool dry season of 2014

Code	Sex	Weight (g)	SVL (mm)
1HH01	F	175	118.63
1HH02	F	200	123.72
1HH03	F	230	126.66
1HH04	F	170	122.83
1HH05	F	160	117.00
1HH06	F	200	122.47
1HH07	F	220	125.70
1HH08	F	220	120.20
1HH09	F	185	122.43
1HH10	F	200	123.37
1HH11	F	205	122.46
1HH12	F	215	124.28
1HH13	F	160	116.50
1HH14	F	135	112.28
1HH15	F	185	118.29
1HH16	F	205	124.20
1HH17	F	250	127.27
1HH18	F	235	124.49
1HH19	F	190	122.86

Table A-1 Morphological data of farm raised adult *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in cool dry season of 2014 (continued)

Code	Sex	Weight (g)	SVL (mm)
1HH20	F	160	113.67
1HH21	M	110	105.63
1HH22	M	145	112.07
1HH23	M	110	105.62
1HH24	M	135	118.96
1HH25	M	115	111.33
1HH26	M	150	113.58
1HH27	M	115	104.78
1HH28	M	155	116.48
1HH29	M	130	109.38
1HH30	M	125	110.19
1HH31	M	100	101.71
1HH32	M	145	117.20
1HH33	M	160	121.14
1HH34	M	100	98.17
1HH35	M	150	114.42
1HH36	M	140	112.37
1HH37	M	120	110.51
1HH38	M	200	124.20
1HH39	M	160	117.36
1HH40	M	150	116.91

Remarks: - Code 1HHXX is a serial number of adult *H. rugulosus* in cool dry season.
- M and F are male and female, respectively.

Table A-2 Morphological data of farm raised juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in cool dry season of 2014

Code	Sex	Weight (g)	SVL (mm)
2HH01	F	175	123.01
2HH02	F	175	121.45
2HH03	F	245	125.33
2HH04	F	180	120.04
2HH05	F	240	125.34
2HH06	F	170	121.83
2HH07	F	280	139.56
2HH08	F	215	125.42
2HH09	F	150	114.45
2HH10	F	160	115.48
2HH11	F	160	118.73
2HH12	F	190	122.52
2HH13	F	145	111.30
2HH14	F	180	115.20
2HH15	F	145	111.78
2HH16	F	200	124.42
2HH17	F	190	118.40
2HH18	F	130	106.63
2HH19	F	140	109.32
2HH20	F	150	112.80
2HH21	M	115	112.69
2HH22	M	110	107.99
2HH23	M	155	114.89
2HH24	M	165	118.21

Table A-2 Morphological data of farm raised juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in cool dry season of 2014 (continued)

Code	Sex	Weight (g)	SVL (mm)
2HH25	M	155	112.25
2HH26	M	160	115.83
2HH27	M	100	109.92
2HH28	M	140	111.30
2HH29	M	135	115.55
2HH30	M	100	101.05
2HH31	M	135	113.95
2HH32	M	160	122.29
2HH33	M	115	110.42
2HH34	M	95	107.19
2HH35	M	100	106.58
2HH36	M	115	109.50
2HH37	M	100	106.20
2HH38	M	105	104.88
2HH39	M	155	120.84
2HH40	M	120	108.56

Remarks: - Code 2HHXX is a serial number of juvenile *H. rugulosus* in cool dry season.

- M and F are male and female, respectively.

Table A-3 Morphological data of farm raised sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in cool dry season of 2014

Code	Sex	Weight (g)	SVL (mm)
3HH01	F	130	110.19
3HH02	F	135	117.06
3HH03	F	155	121.45
3HH04	F	170	116.94
3HH05	F	170	121.50
3HH06	F	200	126.08
3HH07	F	120	111.82
3HH08	F	295	139.51
3HH09	F	110	106.11
3HH10	F	105	106.11
3HH11	F	100	102.25
3HH12	F	120	112.45
3HH13	F	185	120.20
3HH14	F	180	125.02
3HH15	F	125	106.73
3HH16	F	75	95.81
3HH17	F	190	127.16
3HH18	F	140	122.53
3HH19	F	270	120.44
3HH20	M	110	109.19
3HH21	M	190	123.36
3HH22	M	130	111.63
3HH23	M	140	120.49
3HH24	M	115	114.92

Table A-3 Morphological data of farm raised sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in cool dry season of 2014 (continued)

Code	Sex	Weight (g)	SVL (mm)
3HH25	M	150	120.54
3HH26	M	145	116.64
3HH27	M	145	117.06
3HH28	M	120	108.55
3HH29	M	160	111.59
3HH30	M	135	120.54
3HH31	M	140	118.95
3HH32	M	150	117.71
3HH33	M	100	110.89
3HH34	M	165	114.70
3HH35	M	115	109.06
3HH36	M	135	118.65
3HH37	M	115	114.94
3HH38	M	150	114.70
3HH39	M	150	112.39
3HH40	M	170	119.18

Remarks: - Code 3HHXX is a serial number of adult sick *H. rugulosus* in cool dry season.

- M and F are male and female, respectively.

Table A-4 Morphological data of farm raised adult *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in hot dry season of 2014

Code	Sex	Weight (g)	SVL (mm)
1HH01	M	190	124.54
1HH02	M	180	119.21
1HH03	M	155	113.50
1HH04	M	185	120.11
1HH05	M	180	121.47
1HH06	M	210	125.50
1HH07	M	165	118.12
1HH08	M	205	127.20
1HH09	M	195	118.55
1HH10	M	160	121.98
1HH11	M	190	124.69
1HH12	M	195	123.39
1HH13	M	155	121.30
1HH14	M	200	124.38
1HH15	M	155	118.85
1HH16	M	165	117.92
1HH17	M	170	120.98
1HH18	M	155	115.74
1HH19	M	190	123.74
1HH20	M	140	112.71
1HH21	F	390	144.10
1HH22	F	310	133.84
1HH23	F	320	135.04
1HH24	F	320	134.20

Table A-4 Morphological data of farm raised adult *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in hot dry season of 2014 (continued)

Code	Sex	Weight (g)	SVL (mm)
1HH25	F	280	130.10
1HH26	F	285	136.08
1HH27	F	290	132.46
1HH28	F	330	139.54
1HH29	F	270	132.33
1HH30	F	335	138.19
1HH31	F	245	129.56
1HH32	F	375	143.43
1HH33	F	220	123.42
1HH34	F	315	136.11
1HH35	F	415	149.67
1HH36	F	305	134.12
1HH37	F	260	122.89
1HH38	F	250	129.05
1HH39	F	265	132.12
1HH40	F	245	126.81

Remarks: - Code 1HHXX is a serial number of adult *H. rugulosus* in hot dry season.
- M and F are male and female, respectively.

Table A-5 Morphological data of farm raised juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in hot dry season of 2014

Code	Sex	Weight (g)	SVL (mm)
2HH01	M	155	123.21
2HH02	M	145	119.10
2HH03	M	200	124.35
2HH04	M	170	119.27
2HH05	M	195	126.14
2HH06	M	180	121.52
2HH07	M	200	128.21
2HH08	M	170	117.71
2HH09	M	160	117.57
2HH10	M	160	117.52
2HH11	M	130	111.43
2HH12	M	230	130.66
2HH13	M	170	123.09
2HH14	M	100	103.53
2HH15	M	130	111.45
2HH16	M	145	116.23
2HH17	M	125	106.32
2HH18	M	150	116.92
2HH19	M	185	127.16
2HH20	M	150	123.45
2HH21	F	310	135.01
2HH22	F	280	134.25
2HH23	F	225	126.30
2HH24	F	170	118.13

Table A-5 Morphological data of farm raised juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in hot dry season of 2014 (continued)

Code	Sex	Weight (g)	SVL (mm)
2HH25	F	190	123.27
2HH26	F	280	136.78
2HH27	F	225	125.98
2HH28	F	250	130.33
2HH29	F	260	126.23
2HH30	F	230	113.6
2HH31	F	200	121.55
2HH32	F	210	123.14
2HH33	F	210	124.24
2HH34	F	195	119.78
2HH35	F	160	113.52
2HH36	F	250	129.63
2HH37	F	190	121.09
2HH38	F	180	125.23
2HH39	F	270	127.54
2HH40	F	330	137.43

Remarks: - Code 2HHXX is a serial number of juvenile *H. rugulosus* in hot dry season.

- M and F are male and female, respectively.

Table A-6 Morphological data of farm raised adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in hot dry season of 2014

Code	Sex	Weight (g)	SVL (mm)
3HH01	F	150	111.27
3HH02	F	130	112.29
3HH03	F	130	11.09
3HH04	F	100	101.07
3HH05	F	160	117.48
3HH06	F	160	113.43
3HH07	F	260	136.28
3HH08	F	200	125.19
3HH09	M	115	112.41
3HH10	M	90	108.09

Remarks: - Code 3HHXX is a serial number of adult sick *H. rugulosus* in hot dry season.

- M and F are male and female, respectively.

Table A-7 Morphological data of farm raised adult *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in wet season of 2014

Code	Sex	Weight (g)	SVL (mm)
1HH01	M	190	112.73
1HH02	M	180	173.75
1HH03	M	160	195.88
1HH04	M	130	195.54
1HH05	M	160	190.36
1HH06	M	205	200.72
1HH07	M	155	200.81
1HH08	M	150	196.25
1HH09	M	190	198.61
1HH10	M	165	195.55
1HH11	M	200	198.39
1HH12	M	220	202.22
1HH13	M	190	199.29
1HH14	M	195	196.96
1HH15	M	195	192.81
1HH16	M	135	118.94
1HH17	M	170	117.82
1HH18	M	115	114.38
1HH19	M	170	121.74
1HH20	M	150	116.52
1HH21	F	295	134.52
1HH22	F	305	140.12
1HH23	F	310	137.11
1HH24	F	225	134.48

Table A-7 Morphological data of farm raised adult *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in wet season of 2014 (continued)

Code	Sex	Weight (g)	SVL (mm)
1HH25	F	220	123.26
1HH26	F	320	138.41
1HH27	F	295	131.41
1HH28	F	265	125.91
1HH29	F	195	130.80
1HH30	F	290	135.50
1HH31	F	230	131.70
1HH32	F	390	148.19
1HH33	F	225	126.85
1HH34	F	215	127.74
1HH35	F	345	138.85
1HH36	F	190	125.89
1HH37	F	320	136.15
1HH38	F	200	118.35
1HH39	F	210	126.81
1HH40	F	270	128.47

Remarks: - Code 1HHXX is a serial number of adult *H. rugulosus* in wet season.

- M and F are male and female, respectively.

Table A-8 Morphological data of farm raised juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in wet season of 2014

Code	Sex	Weight (g)	SVL (mm)
2HH01	F	280	130.32
2HH02	F	350	140.26
2HH03	F	370	141.50
2HH04	F	365	143.90
2HH05	F	375	146.73
2HH06	F	385	140.93
2HH07	F	310	136.04
2HH08	F	370	142.98
2HH09	F	360	144.94
2HH10	F	310	137.83
2HH11	F	315	136.14
2HH12	F	360	142.57
2HH13	F	325	139.81
2HH14	F	425	144.84
2HH15	F	440	146.22
2HH16	F	320	134.34
2HH17	F	385	141.92
2HH18	F	340	139.57
2HH19	F	320	136.64
2HH20	F	250	128.80
2HH21	M	170	116.45
2HH22	M	180	119.50
2HH23	M	215	124.52
2HH24	M	240	126.90

Table A-8 Morphological data of farm raised juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in wet season of 2014 (continued)

Code	Sex	Weight (g)	SVL (mm)
2HH25	M	195	122.52
2HH26	M	235	127.34
2HH27	M	180	117.87
2HH28	M	200	120.57
2HH29	M	190	122.99
2HH30	M	190	125.95
2HH31	M	205	122.99
2HH32	M	250	130.97
2HH33	M	245	129.76
2HH34	M	165	120.72
2HH35	M	205	118.73
2HH36	M	215	123.56
2HH37	M	195	121.27
2HH38	M	220	124.22
2HH39	M	230	122.70
2HH40	M	205	124.51

Remarks: - Code 2HHXX is a serial number of juvenile *H. rugulosus* in wet season.
- M and F are male and female, respectively.

Table A-9 Morphological data of farm raised adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in wet season of 2014

Code	Sex	Weight (g)	SVL (mm)
3HH01	F	175	121.23
3HH02	F	220	127.18
3HH03	F	235	128.27
3HH04	F	220	130.82
3HH05	F	265	132.85
3HH06	F	80	98.42
3HH07	F	160	116.86
3HH08	F	165	119.04
3HH09	F	140	110.34
3HH10	F	145	108.22
3HH11	F	90	102.15
3HH12	F	120	109.30
3HH13	F	130	110.07
3HH14	M	110	97.80
3HH15	F	120	111.63
3HH16	F	135	112.33
3HH17	M	135	111.18
3HH18	M	105	101.38
3HH19	M	110	105.60
3HH20	F	80	93.25
3HH21	F	90	98.30
3HH22	F	65	95.36
3HH23	F	65	91.58
3HH24	F	75	114.92

Table A-9 Morphological data of farm raised adult sick *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in wet season of 2014 (continued)

Code	Sex	Weight (g)	SVL (mm)
3HH25	F	90	102.92
3HH26	F	55	86.02
3HH27	F	45	87.90
3HH28	M	90	102.05
3HH29	F	60	93.73
3HH30	M	85	95.81
3HH31	F	70	97.78
3HH32	M	100	103.58
3HH33	M	90	103.67
3HH34	F	60	89.41
3HH35	M	60	93.39
3HH36	F	65	92.57

Remarks: - Code 3HHXX is a serial number of adult sick *H. rugulosus* in wet season.

- M and F are male and female, respectively.

Table A-10 Morphological data of *H. rugulosus* collected from Nan Province from July to August 2014

Code	Sex	Weight (g)	SVL (mm)
1WN01	M	95	107.23
1WN02	M	123	104.34
1WN03	M	110	97.23
1WN04	M	90	91.10
1WN05	M	80	91.12
1WN06	M	100	98.10
1WN07	M	90	90.80
1WN08	M	90	90.80
1WN09	M	115	102.88
1WN10	M	80	91.97
1WN11	M	105	105.13
1WN12	M	110	109.80
1WN13	M	95	96.59
1WN14	M	60	87.46
1WN15	M	110	108.37
1WN16	M	80	95.98
1WN17	M	90	100.45
1WN01	F	90	96.78
2WN02	F	120	105.10
2WN03	F	170	116.61
2WN04	F	120	114.15
2WN05	F	100	110.00

Table A-10 Morphological data of *H. rugulosus* collected from Nan Province from July to August 2014 (continued)

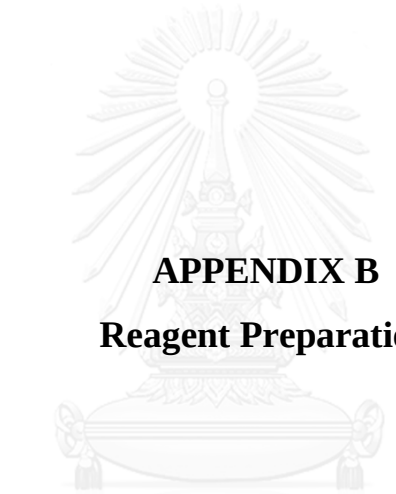
Code	Sex	Weight (g)	SVL (mm)
2WN06	F	110	96.90
2WN07	F	170	118.40
2WN08	F	95	105.99
2WN09	F	150	121.29
2WN10	F	200	128.10
2WN11	F	160	119.65
2WN12	F	70	41.13
2WN13	F	60	87.20
2WN14	F	115	107.01
2WN15	F	125	111.01
2WN16	F	115	106.00

Remarks: - Code 1WNXX and 2WNXX are serial number of male and female *H. rugulosus*, respectively during July to August 2014.

- M and F are male and female, respectively.

APPENDIX B

Reagent Preparation



B-I Phosphate buffered solution pH 7.0 for blood staining

Solution A

Dibasic sodium phosphate, anhydrous (Na_2HPO_4)	9.5 g
Distilled water	1,000 mL

Solution B

Monobasic potassium phosphate (KH_2PO_4)	9.7 g
Distilled water	1,000 mL

Working solution

Solution A	61.1 mL
Solution B	38.9 mL
Distilled water	1,000 mL

Adjust pH to 7.0 and store at 4 °C

B-II Natt and Herrick solution for blood cell count by hemocytometer

Chemicals	1,000 mL	250 mL	100 mL
1. Sodium chloride (NaCl)	3.88 g	0.97 g	0.388 g
2. Sodium sulfate (Na_2SO_4)	2.50 g	0.625 g	0.250 g
3. di-Sodium hydrogen phosphate dodecahydrate (Na_2HPO_4 . $12H_2O$)	2.91 g	0.7275 g	0.291 g
4. Potassium dihydrogen phosphate (KH_2PO_4)	0.25 g	0.625	0.025 g
5. Formaldehyde 37%	7.50 mL	1.875 mL	0.75 mL
6. Methyl violet 2B	0.10 g	0.025 g	0.01 g

Working solution

- Mix all salts and dissolve in 100 mL distilled water
- Transfer the solution to a volumetric flask, add formaldehyde and methyl violet 2B
- Adjust to the required volume (1,000, 250 or 100 mL) in volumetric flask by distilled water

APPENDIX C

Additional Hematological Data

C-I Total leukocyte counts from blood smear of wild caught *H. rugulosus*

The values of total leukocyte counts from blood smear of wild caught *H. rugulosus* were summarized in Table C-1.

Mean total leukocyte count of male frogs (32.24 ± 13.94 cells/300 erythrocytes) was not significantly different from those of the females (23.81 ± 10.70 cells/300 erythrocytes, t = 1.91, df 31, P = 0.06).

Table C-1 Total leukocyte count from blood smear (mean ± SD) of *H. rugulosus* collected from Nan Province in wet season 2014

Parameters	Sex	Mean	SD	Range
Total leukocyte count	Male	32.24	13.94	11.00-61.00
(cells/300	Female	23.81	10.70	10.00-54.00
erythrocytes)	Both sexes	28.15	13.00	10.00-61.00

C-II Blood parasites in wild caught *H. rugulosus*

Thirty-three adult frogs (17 male and 16 female frogs) were collected from a wild population in Nan Province, northern part of Thailand during wet season 2014. For blood parasite examination, thin blood smears were made and stained with Giemsa. The results showed that, 12 frogs (36 % prevalence) were found to be infected by blood parasite, *Hepatozoon* sp (Figure C-1; Sailasuta et al., 2011). The infected female frogs showed significantly higher number of monocyte, neutrophil and eosinophil compared to the non-infected females (Tables C-2). Since parasite infection is thought to be one of the potential threats of population declines in many amphibians, the *Hepatozoon* sp. infection in wild *H. rugulosus* population should be monitored closely.

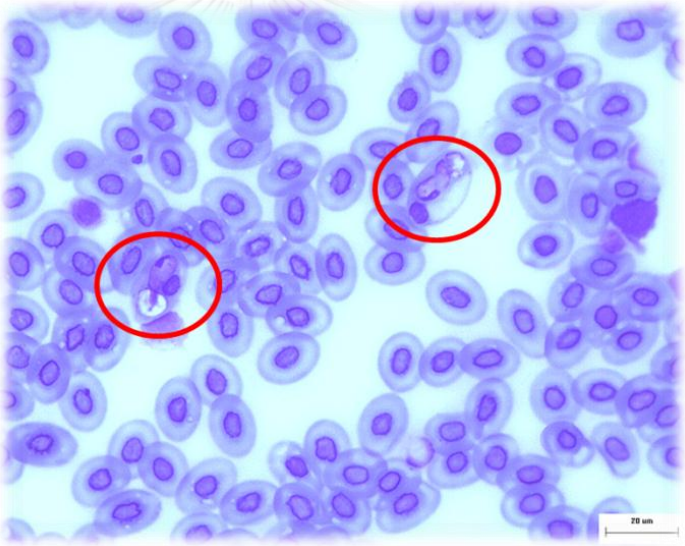


Figure C-1 Light micrograph showing the infection of *Hepatozoon* sp. in erythrocytes (O) of wild caught adult *H. rugulosus* collected from agricultural areas, Wiang Sa District, Nan Province in 2014

Table C-2 Blood cell counts and packed cell volume (mean ± SD) of wild caught *H. rugulosus* infected by *Hepatozoon* sp.

Parameters	Males		Females	
	Non-infected (n=13)	Infected (n=4)	Non-infected (n=8)	Infected (n=8)
Erythrocyte ($\times 10^6$ cells/μL)	0.57±0.30	0.87±0.63	0.66±0.29	0.52±0.26
Leukocyte ($\times 10^3$ cells/μL)	5.37±4.11	5.13±2.68	4.45±1.69	4.03±1.91
PCV (%)	30.00±5.28	33.00±8.73	23.00±5.83	27.19±2.55
Differential lymphocyte (%)	48.43±5.99	47.75±6.20	57.31±8.73	43.65±5.07
Differential monocyte (%)	38.57±7.24	42.64±3.17	32.16±4.12	39.81±6.50*
Differential neutrophil (%)	1.62±1.45	1.25±1.19	0.63±0.74	2.63±2.32*
Differential eosinophil (%)	9.39±2.70	7.63±1.25	8.32±2.28	13.25±4.23*
Differential basophil (%)	0.88±0.63	0.73±0.76	0.53±0.64	0.54±0.78

Remarks: * Significant difference of mean parameter values between infected and non-infected frogs, Student's *t*-test ($P \leq 0.05$)

C-III Total leukocyte count from blood smear of farm raised *H. rugulosus*

The values of total leukocyte count from the smears of peripheral blood of farm raised *H. rugulosus* are summarized in Table C-3 and Figure C-2. In cool dry season, the mean values of total leukocyte count of adult *H. rugulosus* were 13.50 ± 5.40 and 18.80 ± 8.10 cells/300 erythrocytes in males and females, respectively. The mean value of total leukocyte count of juvenile *H. rugulosus* was 18.35 ± 8.89 cells/300 erythrocytes. In hot dry season, the mean values of total leukocyte count of adult *H. rugulosus* were 8.90 ± 5.34 and 9.85 ± 6.29 cells/300 erythrocytes in males and females, respectively. The mean value of total leukocyte count of juvenile *H. rugulosus* was 8.93 ± 4.57 cells/300 erythrocytes. In wet season, the mean values of total leukocyte count of adult *H. rugulosus* were 12.25 ± 5.60 and 9.50 ± 4.49 cells/300 erythrocytes in males and females, respectively. The mean value of total leukocyte count of juvenile *H. rugulosus* was 8.83 ± 3.80 cells/300 erythrocytes.

After seasonal variation analysis (Table C-3, Figure C-2), the result showed that in cool dry season, the mean values of total leukocyte count of adult male *H. rugulosus* was significantly higher than that of the males in hot dry and wet seasons (one-way ANOVA, H = 11.09, df = 2, P = 0.004). The result showed that in cool dry season, the mean values of total leukocyte count of adult female *H. rugulosus* was also significantly higher than that of the females in hot dry and wet seasons (one-way ANOVA, F = 13.31, df = 2, P = <0.001). The mean value of total leukocyte count of juveniles in cool season was also significantly higher than that of the frogs in hot dry and wet seasons (one-way ANOVA, H = 39.31, df = 2, P = <0.001).

Table C-3 Total leukocyte counts values (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Total leukocyte counts (cells/300 erythrocytes)		
	Adult		Juvenile
	Males (n=20)	Females (n=20)	Males & Females (n=40)
Cool dry	13.50 ± 5.40[a]	18.80 ± 8.10 [A]	18.35 ± 8.89 [I]
Hot dry	8.90 ± 5.34 [b]	9.85 ± 6.29 [B]	8.93 ± 4.57 [II]
Wet dry	12.25 ± 5.60 [b]	9.50 ± 4.49 [B]	8.83 ± 3.80 [II]

Remark: Significant difference among seasons (one-way ANOVA, $p<0.05$) is indicated by different superscript letters (small letters for adult males and capital letter for adult females) and roman numerals for juvenile frogs.

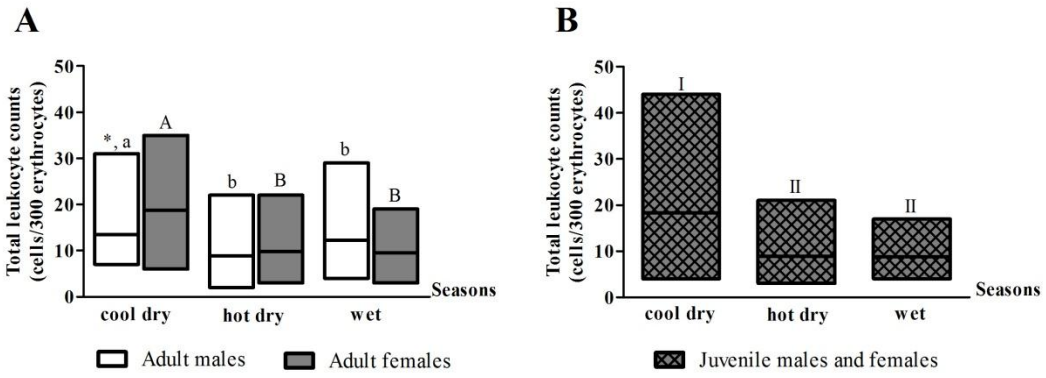


Figure C-2 Total leukocyte counts values (mean and range) of (**A**) adult and (**B**) juvenile of farm raised *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remarks: Significant sex-related difference (Student *t*-test, $p<0.05$) is indicated by an asterisk (*). Significant difference among seasons (one-way ANOVA, $p<0.05$) is indicated by different superscript letters (small letters for adult males and capital letter for adult females) and roman numerals for juvenile frogs.

C-IV Neutrophil to lymphocyte ratio

The values of neutrophil to lymphocyte (N/L) ratio of peripheral blood of farm raised *H. rugulosus* are summarized in Table C-4 and Figure C-3. In cool dry season, the mean value of N/L ratio of adult *H. rugulosus* was 0.64 ± 0.98. The mean values of N/L ratio of juvenile *H. rugulosus* were 1.21 ± 1.12 and 1.54 ± 1.22 in males and females, respectively. In hot dry season, the mean value of N/L ratio of adult *H. rugulosus* was 0.98 ± 1.09. The mean values of N/L ratio of juvenile *H. rugulosus* were 1.22 ± 0.96 and 1.31 ± 1.27 in males and females, respectively. In wet season, the mean value of N/L ratio of adult *H. rugulosus* was 0.33 ± 0.47. The mean values of N/L ratio of juvenile *H. rugulosus* were 1.02 ± 1.09 and 0.39 ± 0.53 in males and females, respectively.

After seasonal variation analysis (Table C-4, Figure C-3), the result showed that in hot dry season, the mean value of N/L ratio of adults *H. rugulosus* was significantly higher than that of the frogs in cool dry and wet seasons (one-way ANOVA, H = 7.96, df = 2, P = 0.019). The result showed that in wet season, the mean value of N/L ratio of juvenile female *H. rugulosus* was also significantly lower than that of the females in cool dry and hot dry seasons (one-way ANOVA, H = 15.61, df = 2, P = <0.001).

Table C-4 Neutrophil to lymphocyte ratio (mean ± SD) of adult and juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Seasons	Neutrophil to lymphocyte ratio		
	Adult	Juvenile	
	Males & Females (n=40)	Males (n=20)	Females (n=20)
Cool dry	0.64±0.98 [b]	1.21±1.12	1.54±1.22 [A]
Hot dry	0.98±1.09 [a]	1.22±0.96	1.31±1.27 [A]
Wet	0.33±0.47 [b]	1.02±1.09	0.39±0.53 [B]

Remark: Significant difference among seasons (one-way ANOVA, $p<0.05$) is indicated by different superscript letters (small letter for adult frogs and capital letter for juvenile females).

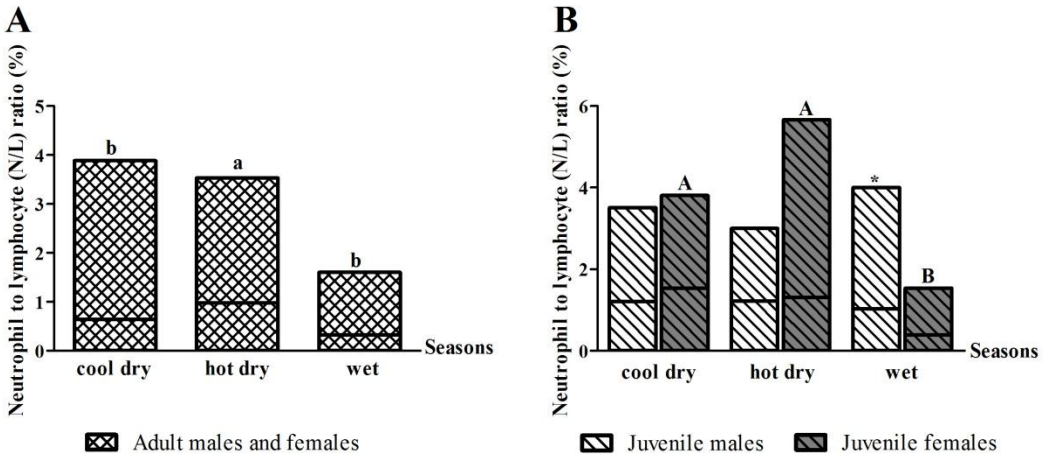


Figure C-3 Neutrophil to lymphocyte ratio (mean and range) of (**A**) adult and (**B**) juvenile *H. rugulosus* collected from the Huai Hong Khrai Royal Development Study Center, Chiang Mai Province in different seasons of 2014

Remarks: Significant sex-related difference (Student *t*-test, $p<0.05$) is indicated by an asterisk (*). Significant difference among seasons (one-way ANOVA, $p<0.05$) is indicated by different superscript letters (small letter for adult frogs and capital letter for juvenile females).

Research Dissemination

Research Article

Meesawat, S., Kitana N., Kitana. J. 2016. Hematology of wild caught *Hoplobatrachus rugulosus* in northern Thailand. *Asian Herpetological Research*, 7: 131-138. [IF 2015 = 0.513]

Poster Presentations (International)

Meesawat, S., Kitana N., and Kitana. J. Health status based on hematological examination of wild-caught rice field frog in Thailand. *Abstract: International Wildlife Management Congress (IWMC)*, July 26-30, 2015, Sapporo, Japan.

Meesawat, S., Kitana N., and Kitana. J. Seasonal hematological profile of the rice field frog *Hoplobatrachus rugulosus* (Wiegmann, 1835). *Abstract: JSPS Core-to-Core Program the 5th International on Asian Vertebrate Species Diversity (AVIS)*, December 15-18, 2015, Bangkok and Saraburi, Thailand.

VITA

Mr. Suthirote Meesawat was born on July 7th, 1989 in Phatthalung Province, Thailand. He has graduated a Bachelor of Science degree in Biology from Department of Biology, Faculty of Science, Chulalongkorn University since 2012. He has then continued his study as a graduate student in Master's degree Program in Zoology, Department of Biology, Faculty of Science, Chulalongkorn University. During his study, he carried out his research at Huai Hong Khrai Royal Development Study Center, Chiang Mai Province, northern part of Thailand and The Chulalongkorn University Forest and Research Station, Lainan Subdistrict, Wiang Sa District, Nan Province for academic year as part CU-ANR-57-01 and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) grants support. During the academic year of his study, he was a teaching assistant (General Biology Laboratory, Basic Anatomy Laboratory, Histology Laboratory, Microtechnique Laboratory, Animal Physiology Laboratory, Field Study in Biology Laboratory and Ecology Laboratory) at Department of Biology, Faculty of Science, Chulalongkorn University. During his graduate practice, he has presented part of his work as poster presentations in the international conferences including 5th International Wildlife Management Congress 2015 at Sapporo, Japan in 2015 and JSPS Core-to-Core Program the 5th International on Asian Vertebrate Species Diversity (AVIS) at Bangkok and Saraburi, Thailand in 2015. In 2015, he has also published a part of his work as a research article in Asian herpetological research, an international research journal listed in ISI.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University