การตอบสนองทางภูมิคุ้มกันของกบหนอง *Fejervarya limnocharis* (Gravenhorst, 1829)
ในพื้นที่เกษตรกรรมที่มีการใช้สารฆ่าวัชพืช ในอำเภอเวียงสา จังหวัดน่าน

นายขัตพันธุ์ จันทะวงษ์ศรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2557

IMMUNE RESPONSE OF RICE FROG *Fejervarya limnocharis* (Gravenhorst, 1829) IN HERBICIDE UTILIZED AGRICULTURAL AREA AT WIANG SA DISTRICT, NAN PROVINCE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
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Thesis Title	IMMUNE RESPONSE OF RICE FROG *Fejervarya limnocharis* (Gravenhorst, 1829) IN HERBICIDE UTILIZED AGRICULTURAL AREA AT WIANG SA DISTRICT, NAN PROVINCE
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ขัตพันธุ์ จันทะวงษ์ศรี : การตอบสนองทางภูมิคุ้มกันของกบหนอง *Fejervarya limnocharis* (Gravenhorst, 1829) ในพื้นที่เกษตรกรรมที่มีการใช้สารฆ่าวัชพืช ในอำเภอเวียงสา จังหวัดน่าน (IMMUNE RESPONSE OF RICE FROG *Fejervarya limnocharis* (Gravenhorst, 1829) IN HERBICIDE UTILIZED AGRICULTURAL AREA AT WIANG SA DISTRICT, NAN PROVINCE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.นพดล กิตนะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร.จิรารัช กิตนะ, 176 หน้า.

การศึกษาอิทธิพลของการใช้สารฆ่าวัชพืชต่อการตอบสนองทางภูมิคุ้มกันของกบหนอง *Fejervarya limnocharis* ที่อาศัยในพื้นที่เกษตรกรรม โดยเก็บตัวอย่างกบหนองจากพื้นที่อ้างอิงที่ไม่มีการใช้สารฆ่าวัชพืช และพื้นที่ปนเปื้อนที่ยังคงมีการใช้สารฆ่าวัชพืช ในจังหวัดน่าน ในปี พ.ศ. 2556 แล้วศึกษาพารามิเตอร์ด้านสุขภาวะและขนาดกับน้ำหนัก ก่อนนำไปศึกษาการตอบสนองทางภูมิคุ้มกันแบบจำเพาะเจาะจงชนิด delayed-type hypersensitivity (DTH) และ แบบไม่จำเพาะเจาะจง ได้แก่ ระดับฮอร์โมนคอร์ติโคสเตอโรน การนับแยกชนิดเม็ดเลือดขาว และจำนวน melanomacrophage และ melanomacrophage center (MMC) ผลการศึกษาพบว่ากบหนองในพื้นที่ปนเปื้อนมีค่าดัชนีสุขภาวะต่ำกว่ากบในพื้นที่อ้างอิงซึ่งแสดงถึงอิทธิพลของสารฆ่าวัชพืชต่อสุขภาวะโดยรวมของกบ มีค่าดัชนีน้ำหนักม้ามสูงกว่ากบจากพื้นที่อ้างอิงซึ่งแสดงถึงอิทธิพลของสารฆ่าวัชพืชต่ออวัยวะในระบบภูมิคุ้มกัน และมีดัชนีน้ำหนักรังไข่สูงกว่ากบจากพื้นที่อ้างอิงในฤดูแล้งหนาวซึ่งแสดงแนวโน้มการกระตุ้นการเติบโตของรังไข่โดยสารฆ่าวัชพืช เมื่อพิจารณาการตอบสนองทางภูมิคุ้มกันแบบจำเพาะเจาะจงพบว่ากบหนองในพื้นที่ปนเปื้อนแสดงแนวโน้มการตอบสนองแบบ DTH ที่ต่ำกว่ากบหนองในพื้นที่อ้างอิงอย่างมีนัยสำคัญ ในขณะที่การตอบสนองทางภูมิคุ้มกันแบบไม่จำเพาะเจาะจงแสดงว่ากบหนองมีระดับฮอร์โมนคอร์ติโคสเตอโรนในน้ำเลือดที่ไม่แตกต่างระหว่างพื้นที่ แต่กบหนองจากพื้นที่ปนเปื้อนมีค่าสัดส่วนเม็ดเลือดขาวชนิดนิวโทรฟิลต่อลิวโคไซต์ต่ำกว่ากบหนองในพื้นที่อ้างอิงอย่างมีนัยสำคัญในช่วงฤดูแล้งหนาวซึ่งเป็นช่วงที่มีการเพาะปลูกในพื้นที่ปนเปื้อนเท่านั้น และยังพบว่ากบหนองในพื้นที่ปนเปื้อนมีจำนวน melanomacrophage และ MMC มากกว่ากบหนองในพื้นที่อ้างอิงอย่างชัดเจน โดยพบว่าจำนวน melanomacrophage และ MMC มีสหสัมพันธ์อย่างมีนัยสำคัญกับปริมาณอาทราซีนในเนื้อเยื่อกบหนอง ซึ่งผลการศึกษาแสดงให้เห็นว่าการใช้สารฆ่าวัชพืชในพื้นที่เกษตรกรรมอาจมีผลเปลี่ยนแปลงภูมิคุ้มกันของกบหนองในพื้นที่ ข้อมูลจากการศึกษาการตอบสนองทางภูมิคุ้มกันของสัตว์สะเทินน้ำสะเทินบกในครั้งนี้จึงช่วยให้หลักฐานยืนยันผลที่พึงระวังจากการใช้สารฆ่าวัชพืชต่อสิ่งมีชีวิตที่ไม่ใช่เป้าหมายในระบบนิเวศทางการเกษตรอันรวมไปถึงมนุษย์

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สัตววิทยา ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5471923723 : MAJOR ZOOLOGY
KEYWORDS: AMPHIBIAN / ATRAZINE / DELAYED-TYPE HYPERSENSITIVITY / HEALTH / MELANOMACROPHAGE CENTER

KHATTAPAN JANTAWONGSRI: IMMUNE RESPONSE OF RICE FROG *Fejervarya limnocharis* (Gravenhorst, 1829) IN HERBICIDE UTILIZED AGRICULTURAL AREA AT WIANG SA DISTRICT, NAN PROVINCE. ADVISOR: NOPPADON KITANA, Ph.D., CO-ADVISOR: JIRARACH KITANA, Ph.D., 176 pp.

This study aims to investigate potential influence of herbicide utilization on immune responses of the rice frog, *Fejervarya limnocharis*, living in agricultural area. Frogs were caught from a paddy field with no history of herbicide utilization (reference site) and a paddy field with intensive herbicide utilization (contaminated site) in Nan Province in 2013. After acclimatization, frogs were examined for health, morphometric and gravimetric parameters before subjected to study for a specific immune response (delayed-type hypersensitivity: DTH) and non-specific immune responses (plasma corticosterone level, differential leukocyte count, and numbers of hepatic melanomacrophage and melanomacrophage center (MMC)). The results showed that frogs from the contaminated site had a significantly lower condition factor indicating potential impact on overall health of frog, a significantly higher relative spleen weight indicating potential effect on immune organ, and a significant increase in ovarian weight in a certain season possibly due to effects of herbicide on ovarian growth. Specific immune response as indicated by the DTH response showed the common trend of significantly lower response in frogs from the contaminated site compared to those of the reference site. The results of non-specific immune response showed that there was no significant site-related difference in plasma corticosterone levels. However, neutrophil / lymphocyte (N:L) ratio was markedly reduced in the contaminated site frog during the cool dry season when agricultural activity was present only at the contaminated site. Numbers of melanomacrophage and MMC were markedly elevated in the contaminated site frogs compared to those of the reference site frogs. Significant correlation between numbers of melanomacrophage and MMC vs. atrazine residue was also evident. Overall results suggest that herbicide utilization in agricultural areas could potentially alter immune function of the populated rice frogs.

Department:	Biology	Student's Signature	.....
Field of Study:	Zoology	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....

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CHAPTER 1
GENERAL INTRODUCTION

Amphibians are animal that inhabit aquatic and semi-aquatic environments on all continents except Antarctica. A recent global amphibian assessment concludes that amphibians are declining more rapidly than either birds or mammals (Stuart et al., 2004). A number of possible factors may contribute to amphibian declines, including overexploitation, habitat destruction, climate change, disease and environmental contaminants (Carey et al., 1999; Stuart et al., 2004). Amphibians are susceptible to pollution because their skin is highly permeable and they have life cycle occurs in both aquatic and terrestrial habitats. Therefore, there are several routes that xenobiotics can enter their body.

Amphibian's response to the contaminants could indicate the impacts on other vertebrates and human (Roy, 2002). Recent evidence suggested that environmental chemicals could suppress immune defenses and magnify the effects of disease (Young et al., 2001). Increase in the animal susceptibility to pathogens may result from stressful conditions due to habitat contamination and subsequent disruption of immune response (Hayes et al., 2010a; Falso et al., 2011). The causes of the decline that frequently reported to involved with each other are habitat contamination and diseases (Hayes et al., 2010a). It is believed that the interactions of contaminants in the habitat and disease may be critical components for the understanding of amphibian declines (Carey et al., 1999).

The immune system plays a crucial role in maintaining health by defending the body against inception of diseases. Amphibian immune defenses involve both innate

defense mechanisms that present before the pathogen is introduced, and adaptive mechanisms that respond specifically to each pathogen. Both natural and man-made disturbances can alter amphibian immune responses. The contaminants could suppress immune defenses and magnify the effects of disease in several animals including amphibians (Carey et al., 1999; Rollins-Smith, 2001; Rohr et al., 2008a; Hayes et al., 2010a).

Amphibian's immune system is believed to be a sensitive target of poisoning by environmental contamination (Rollins-Smith et al., 2007). Some chemicals were reported to affect the immune system of susceptible amphibians. However, there are still a few numbers of reports about alteration in amphibian immune response due to environmental contamination. Some previous report on wildlife has demonstrated contaminant-induced immunosuppression and immunotoxic effects. Examples of these works included effects of pesticides on immunosuppression, increased susceptibility to infections and limb malformations of *Bufo marinus* (*Rhinella marina*) (Linzey et al., 2003), suppressive immune changes and ability to deal with parasitic infection of *Rana pipiens* (Christin et al., 2003; Gilbertson et al., 2003; Christin et al., 2004), sub-lethal alterations of health status of *R. esculenta* (Barni et al., 2007), effects of agricultural activities on *Lithobates catesbeiana* (Falso et al., 2011; Falso, 2011), and effects of environmental cadmium contamination on condition factor, renosomatic index and histopathological changes in the liver and kidney of *Fejervarya limnocharis* (Othman, 2009). It has been suggested that environmental contamination may influence disease emergence by acting directly or indirectly upon the innate or adaptive immune system of the amphibians or by causing disruption in homeostasis.

The rice frog, *F. limnocharis*, is a common amphibian species of South and Southeast Asia that has been regarded as a good sentinel species for environmental contamination (Othman, 2009; Thammachoti, 2012). Justification of using the rice frog as a sentinel species is in accordance with the National Research Council (1991) criteria including 1) it can provide a measurable response to the toxicants, 2) it has a territory or home range that overlaps the area to be monitored, and 3) the easiness to enumerate and capture and sufficient population size and density to permit enumeration.

Agrochemicals have been intensively used for a long time in many agricultural areas of Thailand, especially Nan Province. Previous report on agrochemicals imported to Nan Province showed that 92.04% of agrochemicals are herbicides, mainly atrazine, paraquat and glyphosate (Chanphong, 2008). Agrochemical utilization may lead to the accumulation of its residues in environment and non-target organisms, including human.

Atrazine is not only a well-known endocrine-disrupting chemicals that could affect on reproductive system of amphibians (Kavlock, 2001; Hayes et al., 2002; Hayes et al., 2006; Kloas and Lutz, 2006; Hayes et al., 2010b), but also an immune disruptor of amphibians (Brodkin et al., 2007). Glyphosate and paraquat are found to show adverse effect on amphibians including health and immune impairment (Relyea, 2005; Quassinti et al., 2009; Păunescu and Ponepal, 2011). Since these herbicides have been intensively used in agricultural areas of Nan Province, the frogs that lived in these areas were unavoidably exposed to the herbicides, it is important to examine whether utilization of these herbicides will have any impact on the amphibians living in these areas.

At Nan Province, there are previous reports on herbicide contamination in both environment (atrazine; Maneein et al. (2011), Thammachoti et al. (2012)) and the rice frog tissue (atrazine, paraquat and glyphosate; Thammachoti (2012)). Prior reports showed that herbicide contaminations were found in environment and tissue of the rice frog, *F. limnocharis*, living in paddy fields at Nan Province. Negative impacts on the frog's health, i.e. a significantly lower condition factor in the contaminated site frogs, were evidenced (Thammachoti et al., 2012). This is a sign that the frog was suffered from environmental stress. Therefore, it is interesting to determine the influence of herbicide utilization on the immune system of the rice frogs living in agricultural areas at Nan Province.

Approach of the Study

In this study, the rice frog *F. limnocharis* was used as a sentinel species of environmental health hazards from the herbicide contamination. Since stable population of *F. limnocharis* could be found in the agricultural areas, the species it thus susceptible to long term exposure and accumulation of xenobiotic (Othman, 2009). The rice frog was collected from the agricultural areas with different degree of herbicide utilization and subjected to immune response investigation for multiple parameters (Figure 1-1). Morphomertry and gravimetry of spleen, liver, kidney and gonad as well as the determination of condition factor were carried out. In addition, frog was examined for specific and non-specific immune response using several techniques modified for field study. Association between herbicide residues (Thammachoti, 2012) and number of melanomacrophage and melanomacrophage center (MMC) as a non-specific immune parameter were examined in order to test for

influence of herbicide contamination on immune response of frog as non-target organisms.

Research Objectives

General Objective

To study the immune response of the rice frog *Fejevarya limnocharis* as a sentinel species for herbicide contamination

Specific Objectives

The specific aims of this study are to

1. Compare health, morphometric and gravimetric parameters of the rice frog *F. limnocharis* living in agricultural areas with different degree of herbicide utilization at Wiang Sa District, Nan Province.
2. Compare specific and non-specific immune response of the rice frog *F. limnocharis* living in agricultural areas with different degree of herbicide utilization at Wiang Sa District, Nan Province.
3. Analyze correlation between number of melanomacrophage and melanomacrophage center (MMC) in liver of the rice frog *F. limnocharis* versus herbicide residues in the rice frog, *F. limnocharis*, living in agricultural areas with different degree of herbicide utilization at Wiang Sa District, Nan Province.

Research Hypothesis

There are significant differences in the 1) morphometric and gravimetric indices, 2) specific immune response parameter and 3) non-specific immune response parameters between *Fejervarya limnocharis* from contaminated site with those from reference site.

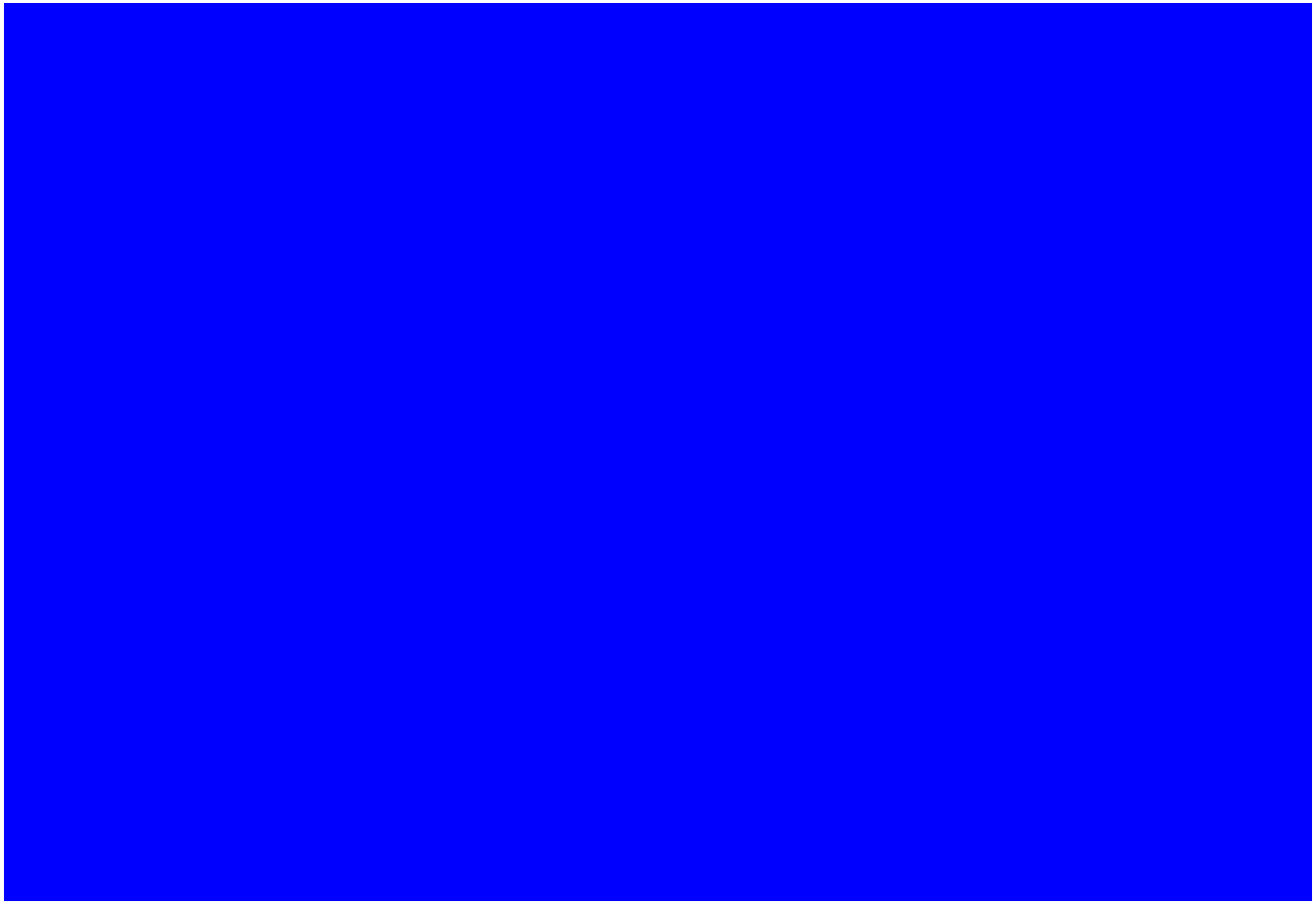


Figure 1-1 Research scheme of this study

CHAPTER 2
LITERATURE REVIEWS

1. Agricultural Activities in Nan Province

Nan Province has a total area of 11,472 km^2 with population of more than 475,000 individuals and is located in the northeastern part of Thailand. Most people of Nan Province are farmer, especially people who live in the low land at central and southern part of the province where Nan River run through the areas. Most of agricultural activities in these areas are generally rice cultivation and backyard gardening (Thadaniti and Prachuabmoh, 2005).

In Wiang Sa District, the southern part of Nan Province, more than 96.7 % of the land have been used for agricultural purposes (Wiang Sa District Agricultural Extension Office (2002); Figure 2-1)

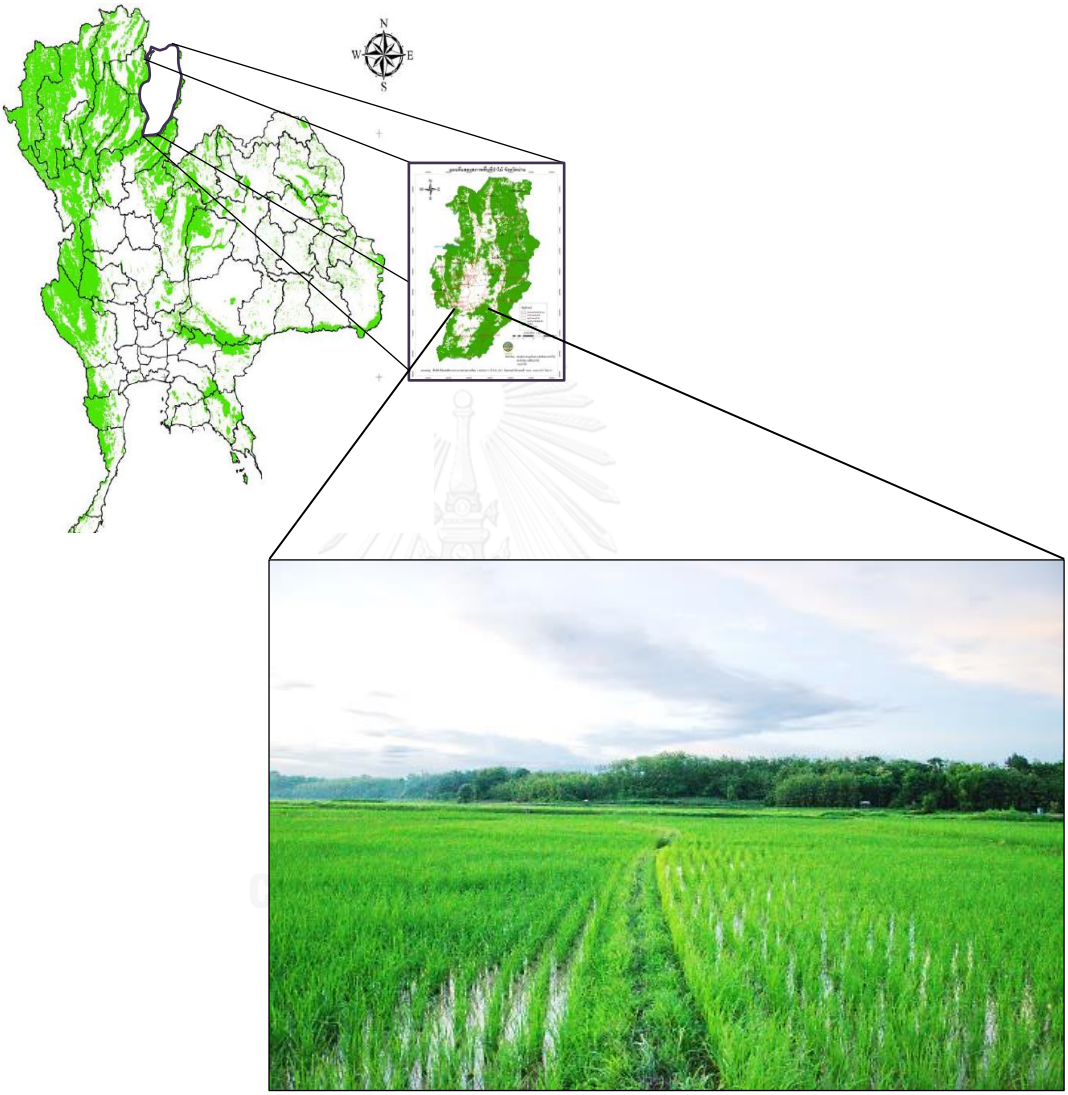


Figure 2-1 Agricultural areas in Nan Province, northern part of Thailand (modified from Forest Land Management Office website: http://www.forest.go.th/fl_mgt/index.php and Thammachoti (2012))

At present, there are many type of cultivation, for example, rice, maize, tobacco, rubber tree, soy bean, orange and litchi orchards at Nan Province (Wiang Sa District Agricultural Extension Office, 2002). Agrochemicals such as pesticides and fertilizers have been intensively used for a long time in these activities. Among these chemicals, more than 92.04% was herbicides (1,172,700 kg), followed by 4.44% (56,600 kg) of fungicides and 3.52% (44,800 kg) of insecticides (Figure 2-2).

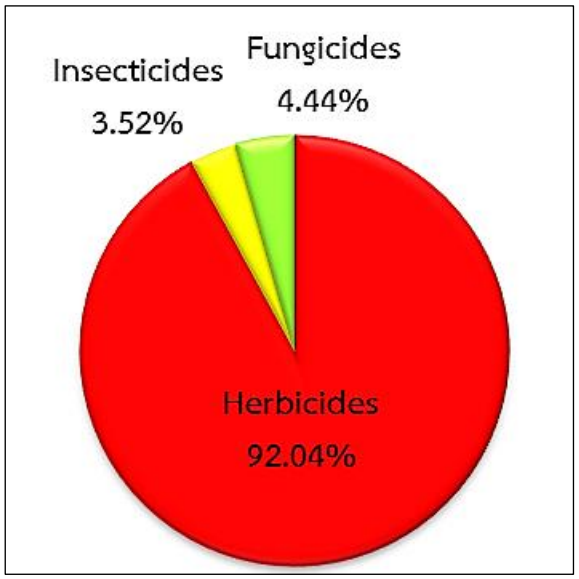


Figure 2-2 Percentage of agrochemical categories imported into Nan Province in 2008 (Chanphong, 2008)

Most of pesticides, especially herbicides, were extensively used in the paddy fields in Nan Province. The most common herbicides used in the paddy fields were glyphosate and paraquat (Panuwet et al., 2012; Wongwichit et al., 2012). Previous study at Nan Province showed that atrazine utilization in the paddy field was also evidenced in addition to glyphosate and paraquat. These three herbicides have been intensively used as herbicide-mixture in the same area or used at different period depending on yearly crop cycle (Thammachoti, 2012). According to previous

study at Nan Province, these herbicides (atrazine, glyphosate and paraquat) could be found in tissue of the rice frog from both contaminated site and reference site with the significant site-related difference in paraquat levels (Thammachoti, 2012). Besides, contaminant analysis of environmental samples showed that atrazine residue was found in Nan River, during the dry season (Maneein et al., 2011; Thammachoti et al., 2012).

2. Herbicides

2.1 Atrazine

2.1.1 Background of Atrazine

Atrazine (6-chloro-N_2-ethyl-N_4-isopropyl-1, 3, 5-triazine-2, 4 diamine; Figure 2-3) is a systemic triazine herbicide. The atrazine herbicides were used to control pre-emergence broad leaf weed and grassy weed. In target plants, atrazine inhibits photosynthesis via competition with plastoquinone II at its binding site in the electron transport process in photosystem II (Devine et al., 1993). Nowadays, atrazine is the second most commonly used pesticide in the United States (Hayes et al., 2002) and perhaps the world (Solomon et al., 1996; van Dijk and Guicherit, 1999). The environmental risk posed by atrazine to aquatic systems is presently being reconsidered by the U.S. Environmental Protection Agency (U.S. EPA, 2003b; U.S. EPA, 2007). One of the challenges in evaluating the safety of atrazine has been that its biological effects are highly controversial, and many of the debates in the literatures have been targeted at its effects on freshwater vertebrates (Hayes, 2004; Renner, 2004). Moreover, atrazine is known as a suspected immunosuppressant (Larson et al., 1998; Kiesecker, 2002; Christin et al., 2003) and endocrine disrupting chemicals (EDCs) that can exert its effect on animals or the non-target organisms (Deb, 2005;

Hayes et al., 2006). U.S. EPA (2003a) reviewed that atrazine has potential developmetal effect on amphibians. Majority of reports on the effect of atrazine on non-target organisms dealed with amphibians (Solomon et al., 2008). As a result of the critical reviews on atrazine toxicity, the maximum contamination level of atrazine is set as low as 0.003 mg/L for drinking water (U.S. EPA, 2009).

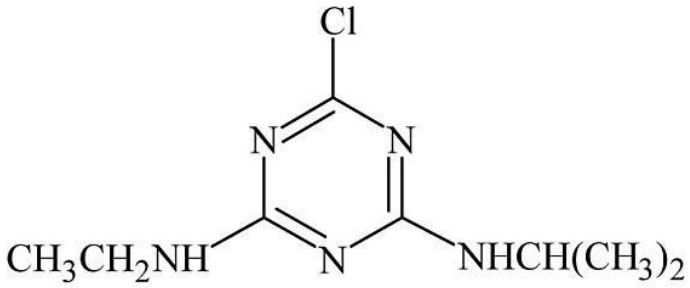


Figure 2-3 The chemical structure of atrazine

2.1.2 Effect of Atrazine on Amphibians Immunity

In the past, atrazine was believed to be toxic to amphibians only at high concentration. However, conflicting evidences have emerged during the past 10 years. Low level of atrazine consistently reduced amphibian size, which is likely to have adverse effects on amphibian populations because smaller metamorphs generally have lower terrestrial survival, lower lifetime reproduction, and compromised immune function (Smith, 1987; Scott, 1994; Carey et al., 1999). However, population-level effects of atrazine have not been empirically tested in nature and thus need to be evaluated explicitly.

According to Rohr and McCoy (2010), atrazine exposure at ecologically relevant concentrations associated with an increase of infection end points. Although high concentrations of atrazine seem to be directly toxic to trematodes and

viruses, possibly reducing infection risk for amphibians (Forson and Storfer, 2006; Koprivnikar et al., 2006; Rohr et al., 2008b), more ecologically relevant concentrations seem to increase amphibian susceptibility, elevating infection risk (Kiesecker, 2002; Gendron et al., 2003; Forson and Storfer, 2006; Rohr et al., 2008c).

Several studies collected data of immunologic effects of atrazine in the animals that also infected with parasites (Kiesecker, 2002; Christin et al., 2003; Gendron et al., 2003; Forson and Storfer, 2006; Hayes et al., 2006; Rohr et al., 2008c). This made it hard to interpret and discriminate the effects of atrazine from the effects of parasite infection. However, in each of these studies, atrazine was proven to be associated with both reduced immune parameters and elevated parasite loads. The elevated infections associated with atrazine cannot be explained by parasite-inducing reduced immune responses. Hence, the parsimonious explanation for both of these findings is that atrazine reduced immune responses, which elevated infections (Raffel et al., 2006). Despite the apparent consistency in the effects of atrazine on immunity and infections, much remains to be learned about the effects of atrazine and other chemicals on parasite–host interactions (Raffel et al., 2006; Raffel et al., 2008).

There were some studies looked at the immunotoxic potential of atrazine in amphibians. According to Brodkin et al. (2007), adult Northern leopard frogs (*Rana pipiens*) exposed to 21 ppb atrazine for 8 days were found to have a decreased innate immune response. A decrease in the number of thioglycollate-elicited peritoneal cavity macrophages was observed in the atrazine-treated frogs (*R. pipiens*) (Brodkin et al., 2007). In addition, these macrophages had decreased phagocytic activity compared to macrophages from the controls (Brodkin et al.,

2007). Another study examined the effect of atrazine on the larvae of Arizona salamanders (*Ambystoma tigrinum*). The larvae exposed to atrazine at environmentally relevant doses had increase susceptibility to *A. tigrinum* virus (Forson and Storfer, 2006). These two studies provide clear evidence that atrazine is toxic to immune system of non-target amphibian species.

Forson and Storfer (2006) also reported that concentrations of 16 µg/L atrazine possibly result in a stress response, which increases corticotropin-releasing hormone, leading to the observed decrease in peripheral leukocytes and an increase in susceptibility to viral infection. Atrazine and pesticide mixtures containing atrazine have been also shown to reduce lymphocyte proliferation in leopard frogs *R. pipiens* (Christin et al., 2003) and circulating eosinophils in wood frogs *R. sylvatica* (Kiesecker, 2002).

2.2 Glyphosate

2.2.1 Background of Glyphosate

Glyphosate (N-phosphonomethyl glycine; Figure 2-4) is an herbicide with trade names as Round up, Rodeo, Shackle, etc. Glyphosate is used to control post-emergence weeds and commonly used as a non-selective herbicide for aquatic weed control in ponds, lakes and canals (Govindarajulu, 2008). Glyphosate is broadly used in agricultural activities to control annual and perennial weeds. Its mode of action is to prevent the synthesis of aromatic amino acids in plants and some microorganisms by inhibiting the enzyme 5-enolpyruvyl shikimate-3-P synthetase (Devine et al., 1993). Most animals do not possess this pathway and obtain the necessary aromatic amino acids from plants and other sources. Because of this,

glyphosate is relatively non-toxic to animals while it is very effective as an herbicide. It is perhaps the most important herbicide ever developed and has been increasingly used in recent years, since it is biodegradable and therefore persists in the environment for only a short time. Because of its low persistence, repeated applications of this herbicide are practiced in agricultural fields and, thereby, large quantities find their way into water bodies. In Thailand, glyphosate is the first rank of imported herbicide in recent years (Office of Agriculture Regulation, 2012). Glyphosate is a weak organic acid comprising of a glycine moiety and a phosphonomethyl moiety. With the relatively low toxicity to non-target organisms, the maximum contamination level of glyphosate is set at 0.7 mg/L in drinking water (U.S. EPA, 2009).

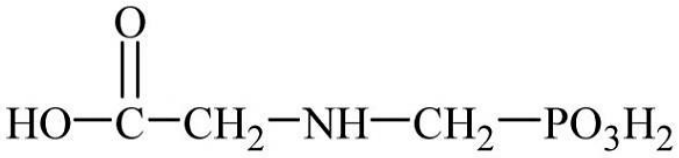


Figure 2-4 The chemical structure of glyphosate

2.2.2 Effect of Glyphosate on Amphibian Immunity

Gahl et al. (2011) reported that exposure of developing wood frogs (*Lithobates sylvaticus*) to glyphosate and chytrid fungus (*Batrachochrytrium dendrobatidis*) was not result in greater susceptibility to chytridiomycosis-induced mortality. On the other hand, the pesticide treatment was found to reduce mortality due to *B. dendrobatidis*.

Glyphosate can impact on amphibians directly and indirectly through several routes. The direct impact includes reducing survival rate, developmental

abnormalities, change in behaviors and also genomic damage, while the indirect impact includes interaction with predators and competitive stress. Physical factors such as temperature, UV radiation, pH and soil could cause synergistic adverse effects of glyphosate in natural condition (Govindarajulu, 2008).

2.3 Paraquat

2.3.1 Background of Paraquat

Paraquat (N, N′-dimethyl-4, 4′-bipyridinium dichloride; Figure 2-5) is one of the most widely used herbicides in the world (Eisler, 1990). Paraquat is mainly formulated as an aqueous solution with surface-active agents. In some countries, a low-strength granular formulation (also containing diquat) is available. Paraquat is a fast-acting, non-selective contact herbicide, absorbed by the leaf with some translocation in the xylem (Lock and Wilks, 2010). Paraquat is a broadspectrum herbicide used to control broad-leaved weeds and grasses in fruit orchards and plantations, and inter-row weeds in many cultivation. It is also used for pre-emergence crops and controlling aquatic weeds. Paraquat adheres to plant surface and exerts its effect by inhibiting the process of photosynthesis and respiration (Haley, 1979). It is rapidly deactivated upon contact with the soil and does not leach (Lock and Wilks, 2010). Mode of action of paraquat in plant includes lipid peroxidation of membranes resulting from superoxide radicals which is similar to the processes in animals (Summers, 1980). The contaminant level of paraquat is set at 0.02 mg/L in drinking water (U.S. EPA, 2009).

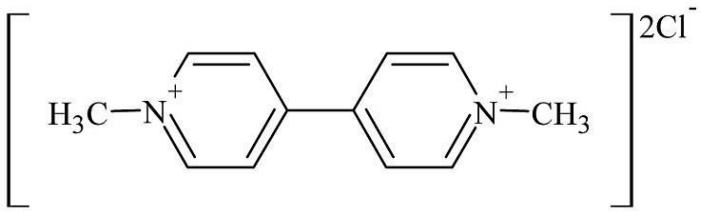


Figure 2-5 Chemical structure of paraquat

2.3.3 Effect of Paraquat on Amphibian Immunity

Study on effect of paraquat-containing pesticide mixture on the American bullfrog (*Lithobates catesbeiana*) showed that plasma glucocorticoid (corticosterone) concentration was not significantly altered after the exposure. Moreover, there was no change in blood oxidative burst activity or neutrophil, lymphocyte, neutrophil to lymphocyte ratios, monocytes, eosinophil, or basophil concentrations observed in this study (Falso, 2011). Other studies about effect of paraquat on amphibians by Dial and Bauer (1984) indicated that growth and swimming behavior of the tadpole of the Northern leopard frog (*Rana pipiens*) could be affected by 0.1 ppm of paraquat. Furthermore, at 5 ppm, paraquat can cause morphological abnormality and reduce growth rate of the tadpoles. Dial and Dial (1987) also found that 2 ppm of paraquat exposure resulted in growth retardation and increase in developmental abnormalities (tail malformation and head defects) in the tadpoles. Eisler (1990) reported that at 0.5 ppm of paraquat, it can affect on survival rate, and developmental abnormalities of the tadpoles. Linder et al. (1990) showed that greater acute and chronic toxicity was found in frogs exposed to a commercial formulation of paraquat (a widely used herbicide) than those exposed to technical grade paraquat.

3. Amphibians As a Sentinel Species for Environmental Contamination

A sentinel species is an organism that used to determine the ecological risk and monitor the environment by providing advance warning of a danger. The terms primarily apply in the context of environmental hazards. Some animals can act as sentinels because they might be more susceptible or have greater chance of exposure to a particular hazard than humans in the same environment (National Research Council, 1991).

According to van der Schalie et al. (1999), the non-human vertebrates could react to an environmental contamination before the impacts could occur to human. The sentinel species could also offer the possibility of expanding our understanding and response to the environmental health concerns.

According to the National Research Council (1991), concepts and definitions of sentinel species include:

1) A sentinel species should have a measurable response to agent(s) in question.
2) A sentinel species should have a territory or home range in the monitored area.
3) A sentinel species should be easily enumerated and captured.
4) A sentinel species must have sufficient population size and density to permit enumeration.

Amphibians were suggested as a good environmental sentinel species for chemical contamination due to their unshelled eggs, semi-permeable skin, life cycle in both aquatic (egg and tadpole) and terrestrial habitats (adult frog), allowing exposure to the pollutant in water or soil during their whole life. Amphibian size is

generally suitable for enumeration and capture, and its population is relatively large. Furthermore, as a member of vertebrates, the measurable effect occurred in amphibian could also occur to other vertebrates including human (Loumbourdis et al., 1999; Roy, 2002).

4. Effect of Pesticides on Morphology of Amphibians

Previous studies have been shown that morphology and population of amphibians could be affected by herbicide contamination. Pesticide contamination can cause deformities in amphibian (Ouellet 2000). The use of pesticides, including herbicides, is the major culprit of wildlife problems, including amphibian declines, because pesticides could affect and disrupt the endocrine systems resulting in deficits in reproduction and development as well as reduced survival rate of the population. Even if with a small amount of endocrine disrupting chemicals (EDCs) exposure, it could cause adverse health effect on these animals in the long run.

Hayes et al. (2006) reported the effect of pesticides on *Rana pipiens* and found that both single atrazine and mixture of pesticides could significantly reduce body weight and length of the frog, indicating that pesticides can affect on morphology of frog living in an agricultural area. McCoy et al. (2008) also found that the cane toad *Bufo marinus* living in an agricultural area showed change in sex hormones and sex organ deformities (e.g. intersex) in relation to different degree of agricultural activities.

Using the rice frog *Fejervarya limnocharis* as a sentinels for herbicide contamination, Thammachoti (2012) described that the frog condition factor was disturbed by the intensive use of herbicides. In addition, the results on

hepatosomatic index and gonadosomatic index indicated that frog from the contaminated site has enlarged liver and enhanced ovarian growth even in the dry period. In addition, it was found that frogs living the potentially contaminated site showed significantly higher level of fluctuating asymmetry (FA) or the deviation from perfect symmetry of the rice frog morphological traits compared to those of the reference site, indicating a higher environmental stress in the herbicide utilization area. These results suggested that the mixed and long term use of herbicides in agricultural area could influence on morphology of the rice frog inhabiting in this area.

In other chemical contamination, Söderman et al. (2007) found that a fluctuating asymmetry of frog living in acid environment was significantly higher than those in a neutral environment indicating the effect of environment on development. In addition, the frog in acid environment was smaller in size and its body was significantly lighter than those in the neutral environment. Moreover, Othman (2009) studied an effect of cadmium contamination on rice frog living in paddy fields and found that cadmium could accumulate in frog tissues, and showed changes in morphology of liver, kidney and gonad of the affected frogs.

5. Amphibian Immunity

Although a limited number of amphibian species have been studied in detail, information about the immune system of the *Xenopus laevis*, and the Mexican axolotl, *Ambystoma mexicanum*, reveals that the amphibian immune system is nearly as complex as that of mammals (Rollins-Smith et al., 2007).

The organs that constitute the adaptive immune system are thymus, spleen, and gut-associated lymphoid tissues. There are also areas of lymphopoiesis in

kidney, liver, mesentery, and gills. Bone marrow is present in some anuran species (Du Pasquier et al., 1989).

Anurans (frogs and toads) possess T-lymphocytes and B-lymphocytes that express rearranging T cell receptors (TCR) and immunoglobulin (Ig) receptors (Du Pasquier et al., 1989). Adaptive immune responses are directed by a well-developed major histocompatibility complex (MHC) (Flajnik and Du Pasquier, 1990; Flajnik and Kasahara, 2001). Three Ig isotypes have been described (IgM, IgY, and IgX) (Flajnik, 2002). MHC-restricted cytotoxic and helper T-cell responses have been characterized (Du Pasquier et al., 1989; Robert et al., 2002).

In addition to these well-characterized adaptive immune defenses, amphibians have an array of innate immune defenses. These include complement-mediated defenses (Nonaka et al., 1998), Natural Killer (NK) cells (Horton et al., 1998), phagocytic cells (Manning and Horton, 1982) and antimicrobial peptides secreted into the gut and skin mucosa (Apponyi et al., 2004; Conlon et al., 2004).

6. Immunotoxic Effect of Environmental Chemicals on Amphibians

Because amphibians are often present in aquatic habitats that are disturbed by agricultural activities, it is likely that they are exposed to a variety of pesticides used to control weeds or insects.

Juvenile *Rana pipiens* and *Xenopus laevis* exposed to environmentally relevant concentrations of six pesticides (atrazine, metribuzin, aldicarb, endosulfane, lindane, and dieldrin) showed immunosuppression. Spleen cell numbers were reduced and phagocytic activity was impaired (Christin et al., 2004).

Lymphocytes from pesticide-treated *R. pipiens* exhibited reduced T-cell proliferation *in vitro* in response to mitogens, and the frogs developed increased

parasitism when challenged with a parasitic nematode *Rhabdias ranae* (Christin et al., 2003). The parasitic worms migrated more rapidly to the lungs and reproduced earlier in pesticide-exposed frogs *R. pipiens* (Gendron et al., 2003).

Adult Woodhouse's toads (*Bufo woodhousii*) treated with malathion and bacterium *Aeromonas hydrophila* were more susceptible than toads that were treated with bacterium alone (Taylor et al., 1999).

Gilbertson et al. (2003) reported that *R. pipiens* treated with malathion, DDT, or dieldrin, the specific IgM antibodies were suppressed in comparison with controls. Oxidative burst products measured in whole blood were also decreased. A delayed-type hypersensitivity (DTH) response was enhanced in pesticide-treated frogs in comparison with control frogs. This pattern of altered immune responses observed in the laboratory was also noted in wild frogs collected in pesticide-exposed locations but not in those collected from pesticide-free locations. It could be suggested that this laboratory results may indicate immunosuppression in natural populations (Gilbertson et al., 2003).

7. The Rice Frog

The rice frog *Fejervarya limnocharis* (Gravenhost, 1829) is regarded as one of the cryptic amphibian species (Zug et al., 2001). This frog is also known as Asian grass frog, common pond frog, field frog, grass frog, Indian rice frog, Indian cricket frog, Boie's wart frog, marsh frog, common pond frog and terrestrial frog (Figure 2-6). The rice frog status is identified as the least concern according to the assessment information of the IUCN Red List Category and Criteria. It is widely distributed, tolerant to a broad range of habitats, and its population is presumably large and

appears to be stable at present. Its size is relatively small with the total length of approximately 42-46 millimeters (mm) (Chan-ard, 2003).

Figure 2-6 The rice frog *Fejervarya limnocharis* (Gravenhost, 1829)

Taylor (1962) and AmphibiaWeb (2012) described the external morphology of this frog species as follows. Body was long with small tubercles and occasional small longitudinal folds. Ventrum are smooth except belly and thighs which are granular posteriorly. Snout is pointed. Distinction of tympanum is half to two third of the eye diameter. Fingers are obtusely pointed and the first figure is longer than second one. Subarticular tubercles are small and very prominent. Tibiotarsal articulation reaches tympanum or nares. Toes are obtuse or with a little swollen tips and have a half webbed characters. Colors of body are grayish brown or olive at above with a V-shaped dark mark between eyes. In some individuals, yellow vertebral stripe is present. A light line is appeared along the calf. Thigh is laterally yellow and

marbled with black. Ventrum is white. A special character in male frog is the throat which is mottled with brown.

Taylor (1962) also mentioned that the rice frog is a small amphibian that is significantly important in Thailand. Naturally captured rice frog was used for human consumption at the higher rate than other amphibians. It also serves as an important link in the natural food web because it is a major food for several kinds of snakes. It inhabits most open wet habitat types, including river floodplains, wet agriculture areas such as rice fields, ditches, marshes, parks, gardens and other habitats. Its breeding and larval development take place in various wetland habitats.

The rice frog is widespread throughout South Asia, Southeast Asia, part of China and in the western Japan (Figure 2-7). In Thailand they are commonly found throughout the country especially in agricultural areas (Taylor, 1962; Chan-ard, 2003).

The classification of the rice frog is as follows:

Kingdom Animalia

Phylum Chordata

Subphylum Vertebrata

Class Amphibia

Superorder Salientia

Order Anura

Family Dicroglossidae

Genus *Fejervarya*

Species *Fejervarya limnocharis* (Gravenhost, 1829)

Figure 2-7 Geographic distribution of rice frog *Fejervarya limnocharis* (modified from the IUCN Red List of Threatened Species website: http://maps.iucnredlist.org/map.html?id=58275 and Thammachoti (2012))

Formerly, there were studies using the rice frog as sentinel species for chemical contamination in agricultural areas. Othman et al. (2009) studied on cadmium contamination in rice frog living in paddy fields in Tak Province, Thailand. The researchers found that cadmium can accumulate in tissues of the rice frog. Later on, it was also reported that cadmium contamination in the tissue of the rice frog may affect the morphology of liver, kidney and gonad. The study suggested that the rice frog could be used as a sentinel species for cadmium contamination in paddy fields.

Wu et al. (2012) studied on the potential toxic effects and contamination of DDT (dichlorodiphenyltrichloroethane) and its metabolites on the rice frog from paddy fields in South China. They found that DDT can be accumulated in the rice frog, especially in liver and egg of the rice frog.

In addition, Thammachoti (2012) studied on the potential effects and contamination of herbicides (atrazine, glyphosate and paraquat) on the rice frog from paddy fields in Wiang Sa District, Nan Province, Thailand. Detectable levels of atrazine, glyphosate and paraquat were found in the frogs from both sites with a significant higher level of paraquat in the contaminated site animals. It was also described that site-related differences in health status, gravimetric parameters and fluctuating asymmetry indicate that herbicide utilization could pose adverse effects at different levels to this sentinel organism.

CHAPTER 3
HEALTH, MORPHOMETRIC AND GRAVIMETRIC PARAMETERS OF RICE FROG *Fejervarya limnocharis* LIVING IN AGRICULTURAL AREAS AT NAN PROVINCE, THAILAND

Introduction

At present, environmental contamination is known as one of environmental problems occurring in all over the world. The contamination can be from both of industrial and agricultural activities. Agrochemical utilization in agricultural activities, especially pesticides, is considered as one of the environmental contamination due to the limit capability of agrochemicals to be degraded in nature. When the pesticides are used intensively and continuously for a long time, the residues can be present in the agricultural areas. Non-target organisms living in contaminated areas are thus susceptible to an exposure with the pesticide residues and an adverse effect from this contamination (Hughes, 1996).

Vertebrates, including human, living in vicinity the agricultural areas can be affected by the pesticide contamination. Since non-mammalian vertebrates share several similar structures and function of their organs to those of other vertebrates and human, monitoring adverse changes in the animal life could be used as an early warning of potential impacts from pesticide contamination to human (National Research Council, 1991).

Amphibians, especially anurans, have been widely used as bio-indicators of environmental contamination in freshwater and terrestrial ecosystems (Vogiatzis and Loumbourdis, 1998; Loumbourdis and Vogiatzis, 2002; Papadimitriou and

Loumbourdis, 2003). Amphibians are regarded as one of a good sentinel species for environmental contamination since they can be exposed to pesticides through their natural habitats include agricultural areas (Khan and Law, 2005). Their life cycle in both aquatic and terrestrial habitats and their semipermeable ventral skin (Papadimitriou and Loumbourdis, 2003) making them susceptible to pesticide exposure through several routes (Duellman and Treub, 1986; Roy, 2002; Quaranta et al., 2009). Morphological studies of amphibian can thus be used to evaluate long-term impact of pesticide contamination on vertebrates (Ouellet 2000; Du Preez et al., 2005; Solomon et al., 2008).

Since the environmental contamination is regarded as one of the major cause of amphibian decline in agricultural areas (Sparling et al., 2001), study on amphibian response to contamination can also serve as contribution to global amphibian decline problem by elucidating whether the decline is due to chemical contamination or synergistic of several factors (Brige et al., 2000; Young et al., 2001; Rohr et al., 2008a; Whittaker et al., 2013).

There are a lot of agricultural areas in Thailand, especially at Nan Province in the Northern part, where pesticides have been intensively used for a long time. Most of pesticides used in this area consist of herbicides namely atrazine, glyphosate and paraquat.

Morphometric and gravimetric data has been a good indicator of the effect of pollution on an organism. Frequently, pollutant has a direct effect on growth of an organism. The condition of the whole body and the liver, as measured with the condition factor (CF) and hepatosomatic index (HSI) could provide information on potential pollution impacts. In fish, condition factor is a useful tool for biologists and

managers to gauge the overall health of their population, and a good indicator of their habitat quality and pollution levels (Craig et al., 2005).

Since stable population of rice frog *Fejervarya limnocharis* could be found in the agricultural areas with intensive herbicide utilization in Nan Province making it susceptible to long-term exposure and accumulation of herbicides (Thammachoti, 2012). In this study *F. limnocharis* was used as a sentinel species for environmental health hazardous from the herbicide contamination. Health, morphometric and gravimetric parameters of the rice frog living in agricultural areas with different degree of herbicide utilization were examined in order to test for influence of herbicide contamination on non-target organisms.

Objective

To compare health, morphometric and gravimetric parameters of the rice frog *F. limnocharis* living in agricultural areas with different degree of herbicide utilization at Wiang Sa District, Nan Province.

Materials and Methods

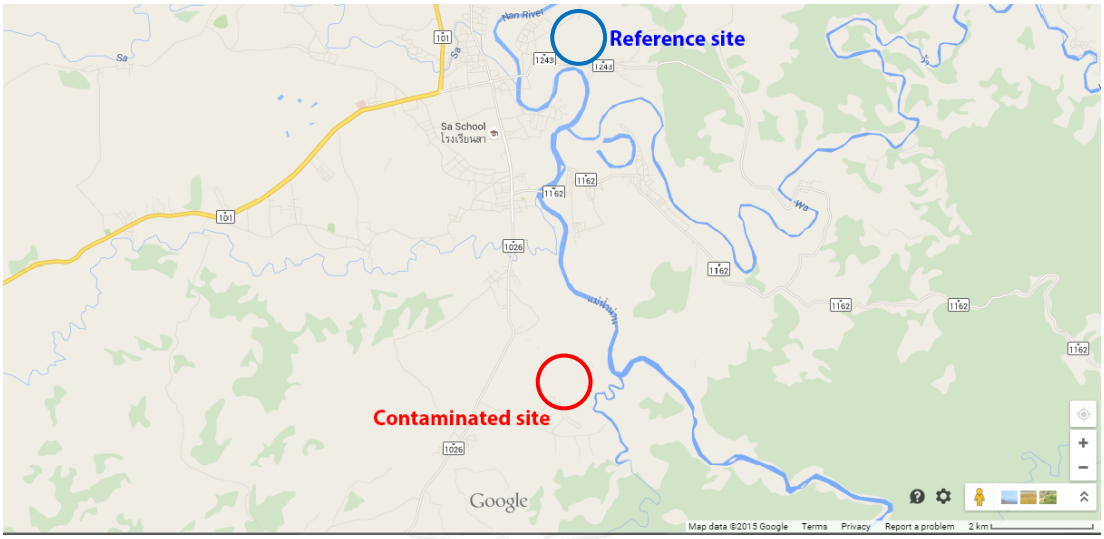
1. Study Sites and Animal Collection

1.1 Study Sites

There are many agricultural areas where herbicides have been intensively used for a long time in Nan Province, particularly in Wiang Sa District. In this study, the two agricultural area in Wiang Sa District, Nan Province were selected as the study sites (Thammachoti, 2012) including:

1) a contaminated site which is an agricultural area with intensive herbicide usage located in San Sub District (47Q 0687729 UTM 2054283)
2) a reference site which is an organic agricultural area with no history of herbicide usage for more than 10 years located in Lai-nan Sub District (47Q 0686779 UTM 2047187)

In general, geographical location, landscape and local climate are similar between both study sites (Figure 3-1). It is believed that the herbicide utilization is the major difference between these two study sites.



Contaminated site
47Q 0687729 UTM 2054283

Reference site
47Q 0686779 UTM 2047187

Figure 3-1 The study sites included a contaminated site which is a paddy field in San Sub District with intensive herbicide utilization and a reference site which is a paddy field in Lai-nan Sub District with no history of herbicide utilization for more than 10 years.
(https://www.google.co.th/maps/search/Wiang+Sa+District+Nan/@18.5477561,100.7531231,13.5z)

Previous study indicated that atrazine residue (0.15 mg/L) was found in the surface water at the contaminated site during late dry season while no herbicide residue was found in the reference site in any season (Maneein et al., 2011; Thammachoti, 2012). Further analyses showed that the frogs from the contaminated site tended to have higher level of glyphosate and markedly higher level of atrazine and paraquat in the tissue compared to those in the reference site (Thammachoti, 2012).

1.2 Sampling Seasons

The samples were divided into three seasons within one year in order to cover range of environmental factors in the region. These sampling periods included:

1) January 2013 (cool dry season)
2) April 2013 (hot dry season)
3) July 2013 – October 2013 (wet season)

Dry and wet seasons in this study were defined based on the climate diagram plot between mean temperature and total rainfall (climograph; Walter et al. (1975)). The climate diagram indicated that the dry season extended from January (cool dry season) in 2013 to April (hot dry season) in 2013 while wet season in this area extended from May to October in 2013 (Figure 3-2).

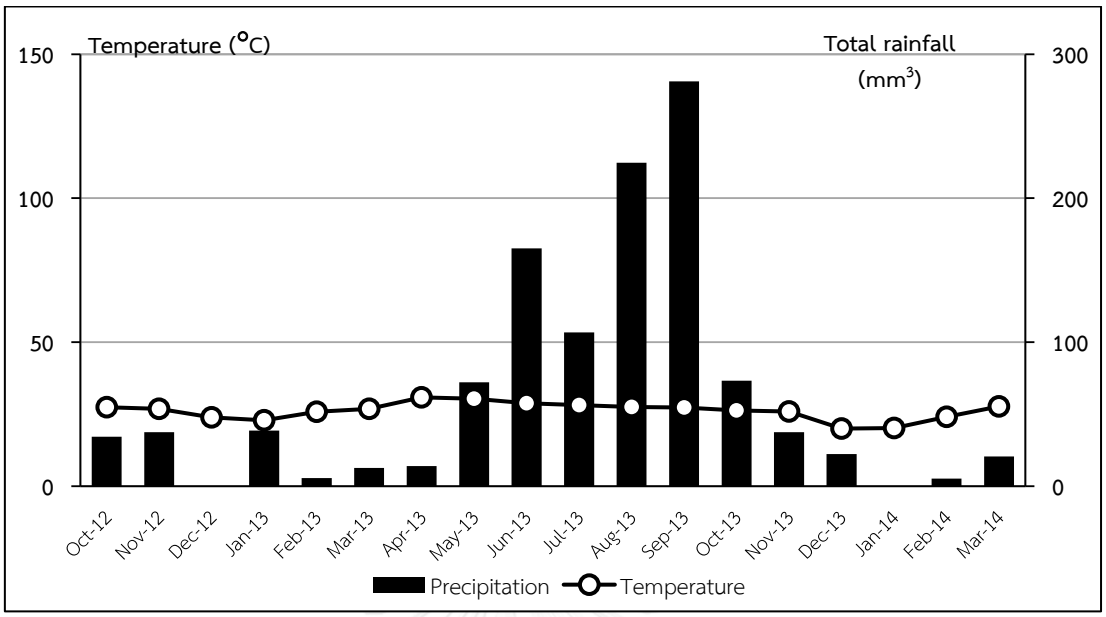


Figure 3-2 The climograph of agricultural areas in Nan Province during sampling periods (January 2013–October 2013)

1.3 Frog Survey and Collection

Frog sample collection was performed 4 times in January, April, July and October in 2013 covering three seasonal period including: cool dry, hot dry and wet seasons. In each month, 40 adult rice frogs (per site) were sampled by hand after visual encounter survey (Crump and Scott, 1994) from contaminated site and reference site. After transportation to a laboratory at the Chulalongkorn University Forest and Research Station, Nan Province, the rice frogs from each site were acclimated for 2 days in plastic aquaria (1 frog per 1 plastic aquarium). During the acclimatization, frogs were fed ad libitum with the small crickets and water in plastic aquaria was changed every 2 days.

1.4 Physical factors

The physical factors of the reference and contaminated sites were recorded during frog sample collection as follows: soil pH was determined by a soil pH and moisture tester, while temperature and relative humidity were measured by a thermo-hygrometer.

2. Morphometric and Gravimetric Analyses

After acclimatization, frogs were euthanatized by immersion into 0.5% Ethyl 3-aminobenzoate methanesulfonate salt (MS-222) solution (Sigma-Aldrich, St. Louis, USA). Snout-vent length (SVL) and body weight (BW) of each frog was recorded, and weight of spleen, liver, kidney and gonad (ovary or testis) of each individual were measured after dissection. These organs were fixed in Davidson's fixative for 48 hrs., and stored in 70% Ethanol for further study. SVL of frog was measured with Mitutoyo Absolute Digimatic caliper (accuracy 0.01 mm) and body and organs was weighed with Ohaus Pioneer Analytical Balances PA214 (accuracy 0.0001 g).

2.1 Overall Health Status

The condition factor (CF) was calculated and used as an indicator of overall health of frog populations (Eastwood and Couture, 2002). It has been successfully used in this frog species (Othman, 2009; Thammachoti, 2012; Hegde and Krishnamurthy, 2014a). Calculation for CF is listed as followed.

First, relationship between logarithm of body weight (BW) and logarithm of snout-vent length (SVL) was determined by a regression analysis (Microsoft Excel) and represented in an equation:

$$\log BW = b \log SVL + \log a$$

Afterward, the condition factor was calculated from the formula:

$$(BW \times 100) / (a \times SVL^{b})$$

In addition to the condition factor, constant b or a, scaling coefficient is also regarded as an indicator of growth pattern of a population of rice frog (Le Cren, 1951; Othman, 2009).

2.2 Gravimetric Analyses

Gravimetric analysis was used to examine change in status of each internal organ. Splenosomatic index (SSI) (Dethloff and Schmitt, 2000; Hadidi et al., 2008; Rohlenová et al., 2011), hepatosomatic index (HSI) (Loumbourdis et al., 1999), renosomatic index (RSI) (Deane and Woo, 2007), and gonadosomatic index (GSI) (Goodwin et al., 1992; Dethloff and Schmitt, 2000; Tilton et al., 2003; Maitra et al., 2007; McCallum and Trauth, 2007) are the ratio of organ mass to total tissue mass which were determined from relative weight by these formulas:

Splenosomatic index (SSI) = (spleen weight x 100 / body weight)

Hepatosomatic index (HSI) = (liver weight x 100 / body weight)

Renosomatic index (RSI) = (kidney weight x 100 / body weight)

Gonadosomatic index (GSI) = (gonad weight x 100 / body weight)

3. Statistical Analyses

All parameters were tested for normal distribution (the Kolmogorov-Smirnov test) and homogeneity of variance. The condition factor, splenosomatic, hepatosomatic, renosomatic and gonadosomatic indices was compared between sites 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 between seasons by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls methods for the data are normally distributed. However, if the data are not normally distributed, all parameters were compared between seasons by Kruskal-Wallis Analysis of Variance on Ranks follows by Dunn's Method. Sigma Plot 11.0 was used as statistical software for every tests except the regression analysis and the slope comparison.

Results

1. Physical Factors in Study Sites

The physical factors of the study sites including soil pH (5.80-7.00), temperature (20.0-27.5 °C) and relative humidity (70.0%-91.5%), were not significantly different between sites. Apart from herbicide utilization, physical factors and agricultural activities were similar at these two study sites.

2. Overall Health Status

The average scaling coefficients of rice frog *F. limnocharis* caught from contaminated and reference sites are shown in Figure 3-3. The scaling coefficient of frog caught from contaminated site (3.1379) is lower than frog caught from reference site (3.3430) (Table 3-1). The difference in the scaling coefficient could indicate that there are differences in growth pattern between these populations.

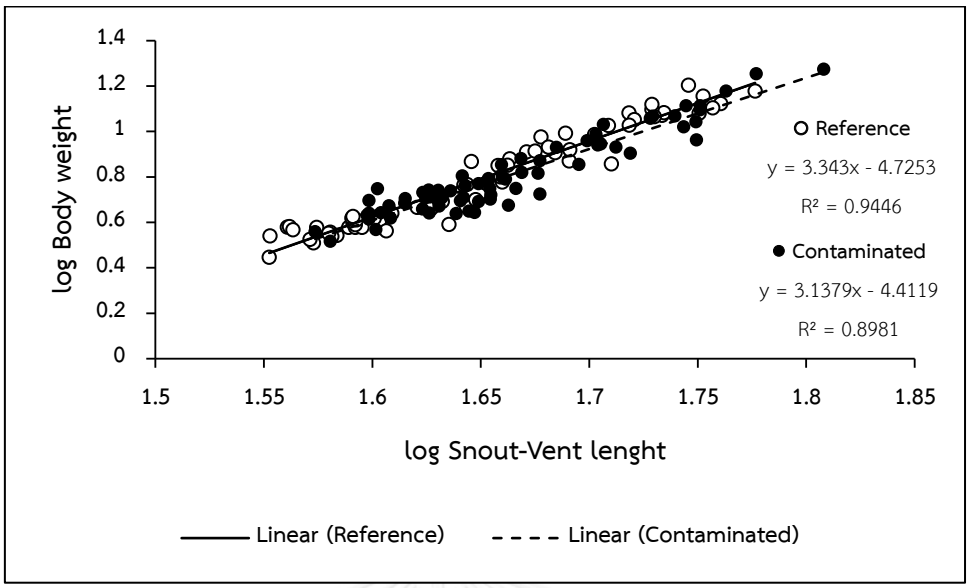


Figure 3-3 Regression analyses of relationship between body weight and snout-vent length of rice frog *Fejervarya limnocharis* from reference and contaminated agricultural areas at Nan Province, Thailand

Table 3-1 Scaling coefficients of rice frog *Fejervarya limnocharis* population caught from reference and contaminated sites at Nan Province, Thailand

	Contaminated site	Reference site
Scaling coefficient	3.1379	3.3430

Furthermore, the condition factor (CF) of rice frog *F. limnocharis* caught from contaminated and reference sites are shown in Figure 3-4. It was found that the overall condition factor of rice frogs caught from reference site (101.87 ± 1.47) was significantly higher than the frog caught from contaminated site (96.28 ± 1.46) (Student t-test, $p<0.05$; Table 3-2).

To accommodate statistical analysis, frogs were grouped according to sampling period into 3 groups including January 2013 (cool dry season), April 2013 (hot dry season) and July 2013–October 2013 (wet season). It was found that there were significant site-related differences in condition factor in most cases, if not all, of the sampling periods (January 2013 and July 2013–October 2013). In the reference site, the condition factor in January 2013 were significantly lower than those in April 2013 and July 2013–October 2013 (Table 3-2; 98.04 ± 1.82 vs. 106.56 ± 2.43 and 101.12 ± 2.90; one-way ANOVA, $p<0.05$). In the contaminated site, the condition factor in April 2013 was significantly higher than those in January 2013 and July 2013–October 2013 (Table 3-2; 104.08 ± 2.19 vs. 90.90 ± 2.51 and 93.78 ± 2.09; one-way ANOVA, $p<0.05$).

Table 3-2 Condition factors (Mean ± S.E.M.) of rice frog *Fejervarya limnocharis* population caught from reference and contaminated paddy fields at Nan Province, Thailand

Seasons	Month/year	Condition factor of the rice frogs	
		Contaminated site	Reference site
Cool dry	Jan 2013	90.90 ± 2.51 [b] (N=19)	98.04 ± 1.82 [*, B] (N=21)
Hot dry	April 2013	104.08 ± 2.19 [a] (N=22)	106.56 ± 2.43 [A] (N=21)
wet	Jul–Oct 2013	93.78 ± 2.09 [b] (N=28)	101.12 ± 2.90 [*, AB] (N=24)
Overall	Jan-Oct 2013	96.28 ± 1.46 (N=69)	101.87 ± 1.47 [*] (N=66)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).
- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

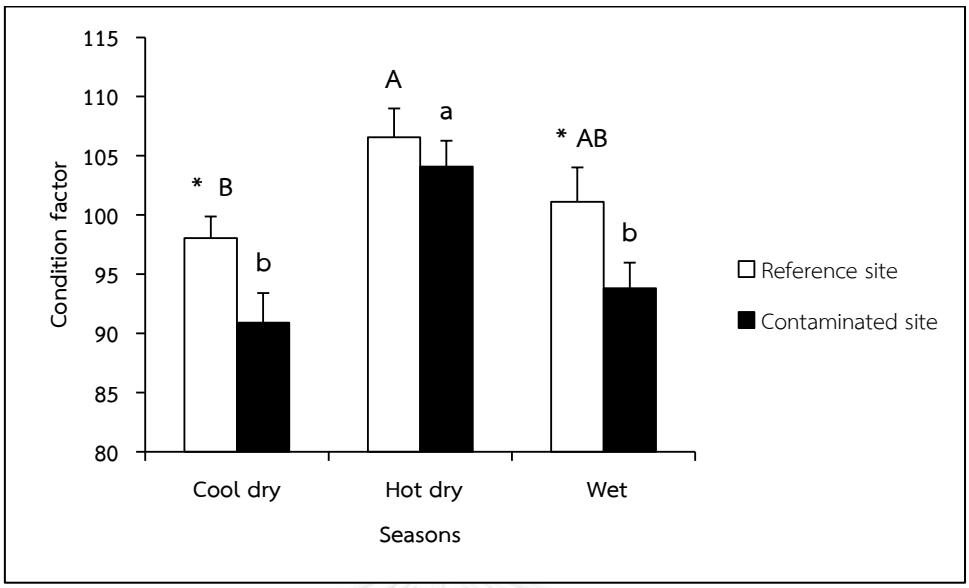


Figure 3-4 Condition factors (Mean ± S.E.M.) of rice frog *Fejervarya limnocharis* from reference and contaminated agricultural areas at Nan Province, Thailand

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

3. Gravimetric Analysis

3.1 Splenosomatic Index (SSI)

There was significant difference between splenosomatic index in male and female frog (Student t-test or Mann-Whitney rank sum test, $p<0.05$). Therefore the data between male and female was analyzed separately.

Overall splenosomatic index of male frog caught from reference site (0.054 ± 0.01) was significantly lower than the male frog caught from contaminated site (0.096 ± 0.02) (Mann-Whitney rank sum test, $p<0.05$; Table 3-3). However, there was no site-related difference in overall splenosomatic index of female frog (Table 3.4; contaminated site = 0.055 ± 0.01 vs. reference site = 0.052 ± 0.01; Mann-Whitney rank sum test, $p>0.05$)

Comparing in each periods showed the significant site-related difference in male SSI was found in hot dry season (April 2013) (Table 3-3; contaminated site = 0.068 ± 0.01 vs. reference site = 0.028 ± 0.01; Student t-test, $p<0.05$), while female SSI was significantly different in cool dry season (January 2013) (Table 3-4; contaminated site = 0.07 ± 0.01 vs. reference site=0.04 ± 0.01, $p<0.05$).

The splenosomatic index of male frog was significantly different between sampling periods at each site (Figure 3-5). In the contaminated site, the splenosomatic index in wet season was significantly higher than those in cool dry and hot dry seasons (Table 3-3; 0.096 ± 0.02 vs. 0.068 ± 0.02 and 0.068 ± 0.01; one-way ANOVA followed by Student-Newman-Keuls' multiple comparison, $p<0.05$). In the reference site, the splenosomatic index in wet season were significantly higher than in hot dry season (Table 3-3; 0.079 ± 0.02 vs. 0.028 ± 0.01; Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's Method, $p<0.05$).

The splenosomatic index of female frog showed significant seasonal-related difference between sampling periods at each site (Figure 3-6). In the reference site, the splenosomatic index in wet season was significantly higher than those in cool dry and hot dry seasons (Table 3-4; 0.082 ± 0.01 vs. 0.040 ± 0.01 and 0.024 ± 0.004; Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's Method, $p<0.05$. In the contaminated site, the splenosomatic index in cool dry season and wet season were significantly higher than in hot dry period (Table 3-4; 0.070 ± 0.01 and 0.061 ± 0.01 vs. 0.036 ± 0.01; one-way ANOVA followed by Student-Newman-Keuls' multiple comparison, $p<0.05$).

Table 3-3 Splenosomatic index (Mean ± S.E.M.) of male rice frog *Fejervarya limnocharis* population caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Splenosomatic index of male frogs	
		Contaminated site	Reference site
Cool dry	Jan 2013	0.068 ± 0.02 [b] (N=10)	0.054 ± 0.01 [AB] (N=14)
Hot dry	April 2013	0.068 ± 0.01 [*, b] (N=11)	0.028 ± 0.01 [B] (N=10)
wet	Jul–Oct 2013	0.096 ± 0.02 [a] (N=15)	0.079 ± 0.02 [A] (N=10)
Overall	Jan-Oct 2013	0.09 ± 0.01 [*] (N=36)	0.054 ± 0.01 (N=34)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Mann-Whitney rank sum test, $p<0.05$).
- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison or Kruskal-Wallis One Way Analysis of Variance on Ranks, $p<0.05$; followed by Dunn's Method).

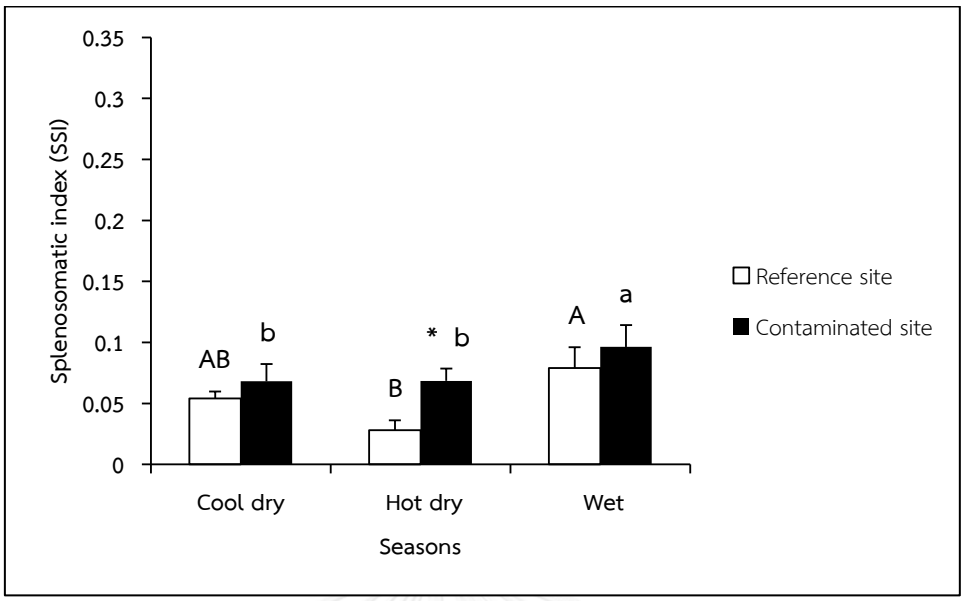


Figure 3-5 Splenosomatic index (Mean ± S.E.M.) of male frogs *Fejervarya limnocharis* from reference and contaminated agricultural areas at Nan Province, Thailand

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Mann-Whitney rank sum test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison or Kruskal-Wallis One Way Analysis of Variance on Ranks, $p<0.05$; followed by Dunn's Method).

Table 3-4 Splenosomatic index (Mean ± S.E.M.) of female rice frog *Fejervarya limnocharis* population caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Splenosomatic index of female frogs	
		Contaminated site	Reference site
Cool dry	Jan 2013	0.070 ± 0.01 $^{*, a}$ (N=9)	0.040 ± 0.01 ^{B} (N=7)
Hot dry	April 2013	0.036 ± 0.01 ^{b} (N=11)	0.024 ± 0.004 ^{B} (N=11)
wet	Jul–Oct 2013	0.061 ± 0.01 ^{a} (N=13)	0.082 ± 0.01 ^{A} (N=14)
Overall	Jan-Oct 2013	0.055 ± 0.01 (N=33)	0.052 ± 0.01 (N=32)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison or Kruskal-Wallis One Way Analysis of Variance on Ranks, $p<0.05$; followed by Dunn's Method).

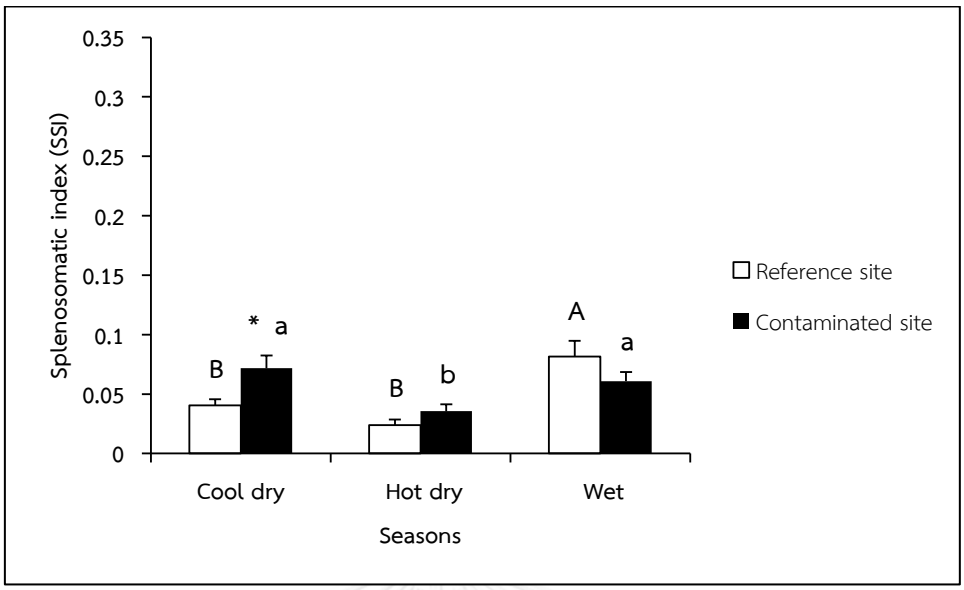


Figure 3-6 Splenosomatic index (Mean ± S.E.M.) of female frogs *Fejervarya limnocharis* from reference and contaminated agricultural areas at Nan Province, Thailand

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison or Kruskal-Wallis One Way Analysis of Variance on Ranks, $p<0.05$; followed by Dunn's Method).

3.2 Hepatosomatic Index (HSI)

Since, there was no significant difference between hepatosomatic index in male and female frog (Student t-test or Mann-Whitney rank sum test, $p>0.05$). Therefore the data between male and female was pooled and analyzed. The overall hepatosomatic index showed no significant difference in the contaminated site frogs (2.74 ± 0.19) compared to those of the reference site (2.69 ± 0.12) (Mann-Whitney Rank Sum Test, $p>0.05$).

It was found that in the reference site, there was significant difference between sampling periods (Figure 3-7). The hepatosomatic index in wet season and hot dry season were significantly higher than in cool dry season (Table 3-5; 3.22 ± 0.21 and 2.75 ± 0.20 vs. 2.03 ± 0.07; one-way ANOVA followed by Student-Newman-Keuls' multiple comparison, $p<0.05$).

Table 3-5 Hepatosomatic index (Mean ± S.E.M.) of rice frog *Fejervarya limnocharis* population caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Hepatosomatic index of frogs	
		Contaminated site	Reference site
Cool dry	Jan 2013	2.86 ± 0.63 (N=19)	2.03 ± 0.07 [B] (N=21)
Hot dry	April 2013	2.53 ± 0.14 (N=22)	2.75 ± 0.20 [A] (N=21)
wet	Jul–Oct 2013	2.83 ± 0.20 (N=28)	3.22 ± 0.21 [A] (N=24)
Overall	Jan-Oct 2013	2.74 ± 0.19 (N=69)	2.69 ± 0.12 (N=66)

Remarks:

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

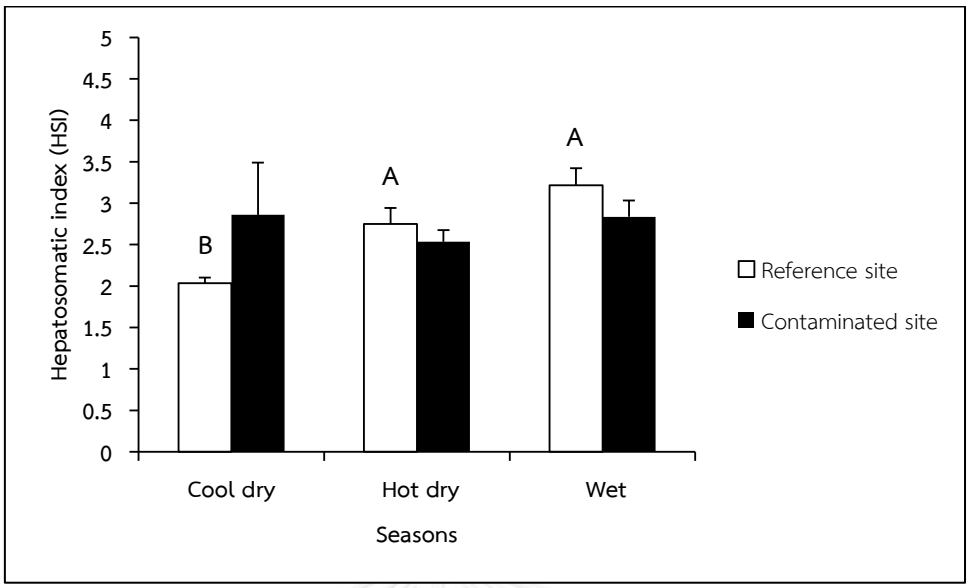


Figure 3-7 Hepatosomatic index (Mean ± S.E.M.) of frogs *Fejervarya limnocharis* from reference and contaminated agricultural areas at Nan Province, Thailand

Remarks:

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

3.3 Renosomatic index (RSI)

Since, there was no significant difference between renosomatic index in male and female frog (Student t-test or Mann-Whitney rank sum test, $p>0.05$). Therefore the data between male and female was pooled and analyzed (Figure 3-8).

The overall renosomatic index showed no significant difference in the contaminated site frogs (0.42 ± 0.01) compared to those of the reference site (0.44 ± 0.01) (Table 3-6; Mann-Whitney Rank Sum Test, $p>0.05$). However, the significant site-related difference in renosomatic index was found in wet season when renosomatic index of rice frogs caught from reference site (0.52 ± 0.02) was significantly higher than the frog caught from contaminated site (0.43 ± 0.01) (Student t-test, $p<0.05$; Table 3-6).

No significant seasonal-related difference in renosomatic index was found in contaminated site (one-way ANOVA, $p>0.05$). On the other hand, the renosomatic index of frogs caught from reference site was significantly different between sampling periods. It was found that in the reference site, the renosomatic index in wet season was significantly higher than those in cool dry and hot dry seasons (Table 3-6; 0.52 ± 0.02 vs 0.40 ± 0.01 and 0.39 ± 0.01; one-way ANOVA followed by Student-Newman-Keuls' multiple comparison, $p<0.05$).

Table 3-6 Renosomatic index (Mean ± S.E.M.) of rice frog *Fejervarya limnocharis* population caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Renosomatic index of frogs	
		Contaminated site	Reference site
Cool dry	Jan 2013	0.43 ± 0.02 (N=19)	0.40 ± 0.01 [B] (N=21)
Hot dry	April 2013	0.40 ± 0.01 (N=22)	0.39 ± 0.01 [B] (N=21)
wet	Jul–Oct 2013	0.43 ± 0.01 (N=28)	0.52 ± 0.02 [*, A] (N=24)
Overall	Jan-Oct 2013	0.42 ± 0.01 (N=69)	0.44 ± 0.01 (N=66)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).
- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

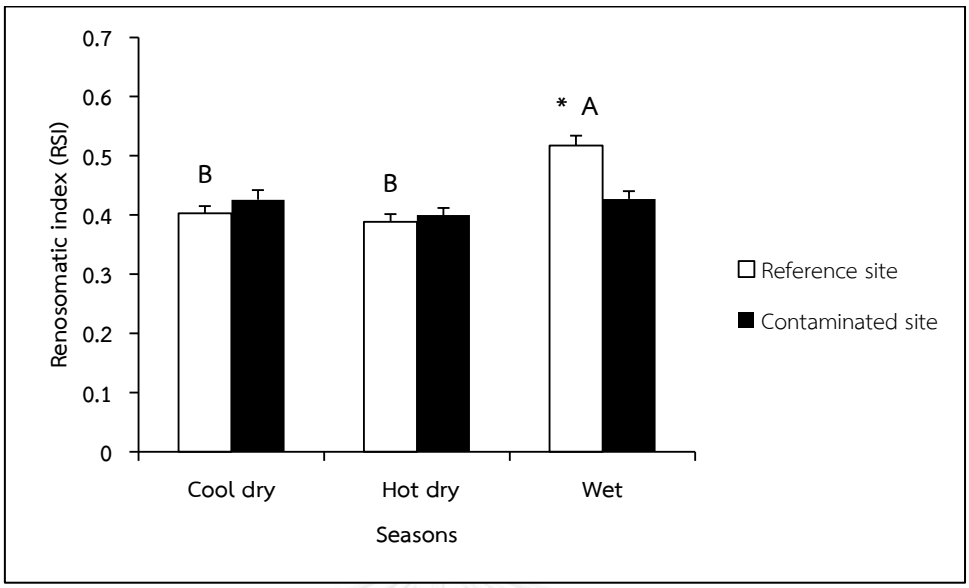


Figure 3-8 Renosomatic index (Mean ± S.E.M.) of frogs *Fejervarya limnocharis* from reference and contaminated agricultural areas at Nan Province, Thailand

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

3.4 Gonadosomatic index (GSI)

The overall gonadosomatic index of male frogs (testicular weight, Table 3-7) in the contaminated site was significantly higher than those of the reference site frogs (0.19 ± 0.01 vs. 0.17 ± 0.01; Mann-Whitney Rank Sum Test, $p< 0.05$). Pairwise comparison in each sampling period also showed similar trend of significantly higher GSI in male frogs from the contaminated site. In hot dry period, gonadosomatic index of male rice frogs caught from the contaminated site (0.21 ± 0.01) was significantly higher than frogs caught from the reference site (0.14 ± 0.01) (Student t-test, $p<0.05$; Table 3-7).

The gonadosomatic index of male frog was significantly different between sampling periods only in reference site (Figure 3-9). In these site, the gonadosomatic indices in wet season was significantly higher than those in cool dry and hot dry periods (Table 3-7; 0.21 ± 0.02 vs. 0.15 ± 0.01 and 0.14 ± 0.01; one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison). No seasonal-related difference in gonadosomatic index was found in the contaminated site frogs (one-way ANOVA, $p>0.05$).

Significant site-related difference in the overall ovarian weight (Table 3-8; contaminated site = 5.49 ± 0.85, reference site = 3.97 ± 0.84; Mann-Whitney Rank Sum Test, $p>0.05$) was found in these frog populations.

However, the markedly site-related difference in gonadosomatic index of female frog was found in cool dry period when GSI of female rice frogs from contaminated site (8.15 ± 0.82) was significantly higher than those of the frog from reference site (1.60 ± 0.16) (Mann-Whitney Rank Sum Test, $p<0.05$; Table 3-8).

The gonadosomatic index of female frog was significant different between sampling periods at each site (Figure 3-10). In the reference site, the gonadosomatic indices in hot dry period was significantly higher than in wet season (Table 3-8; 9.28 ± 1.37 vs. 0.98 ± 0.28; Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's Method, $p<0.05$), while there was no significant difference in cool dry period compared to those in hot dry period and wet season (Table 3-8; 1.60 ± 0.16 vs. 9.28 ± 1.37. and 0.98 ± 0.28; Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's Method, $p>0.05$). In the contaminated site, the gonadosomatic indices in cool and hot dry periods were significantly higher than in wet season (Table 3-8; 8.15 ± 0.82 and 7.43 ± 1.54 vs. 1.85 ± 1.02; Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's Method, $p<0.05$).

Table 3-7 Relative testicular weight (Mean ± S.E.M.) of rice frog *Fejervarya limnocharis* population caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Gonadosomatic index of male frogs	
		Contaminated site	Reference site
Cool dry	Jan 2013	0.15 ± 0.02 (N=10)	0.15 ± 0.01 ^{B} (N=14)
Hot dry	April 2013	0.21 ± 0.01 * (N=11)	0.14 ± 0.01 ^{B} (N=10)
wet	Jul–Oct 2013	0.21 ± 0.02 (N=15)	0.21 ± 0.02 ^{A} (N=10)
Overall	Jan-Oct 2013	0.19 ± 0.01 * (N=36)	0.17 ± 0.01 (N=34)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test or Mann-Whitney Rank Sum Test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

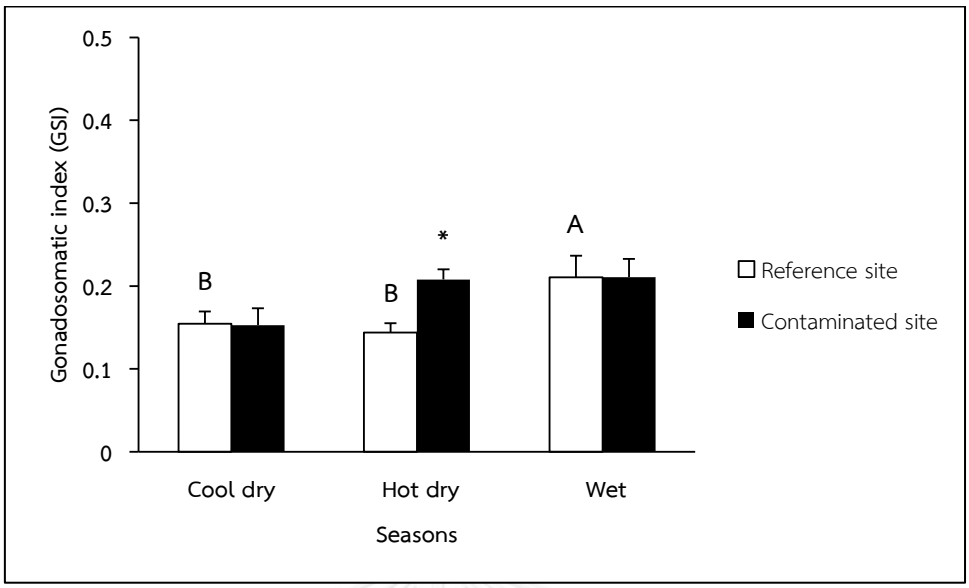


Figure 3-9 Relative testicular weight (Mean ± S.E.M.) of frogs *Fejervarya limnocharis* from reference and contaminated agricultural areas at Nan Province, Thailand

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test or Mann-Whitney Rank Sum Test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

Table 3-8 Relative ovarian weight (Mean ± S.E.M.) of rice frog *Fejervarya limnocharis* population caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Gonadosomatic index of female frogs	
		Contaminated site	Reference site
Cool dry	Jan 2013	8.15 ± 0.82 $^{*, a}$ (N=9)	1.60 ± 0.16 ^{AB} (N=7)
Hot dry	April 2013	7.43 ± 1.54 ^{a} (N=11)	9.28 ± 1.37 ^{A} (N=11)
wet	Jul–Oct 2013	1.85 ± 1.02 ^{b} (N=13)	0.98 ± 0.28 ^{B} (N=24)
Overall	Jan-Oct 2013	5.49 ± 0.85 (N=33)	3.97 ± 0.84 (N=32)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Mann-Whitney Rank Sum Test, $p<0.05$).
- Difference in superscript letter indicates significant difference between sampling periods within the same site (Kruskal-Wallis One Way Analysis of Variance on Ranks, $p<0.05$; followed by Dunn's Method).

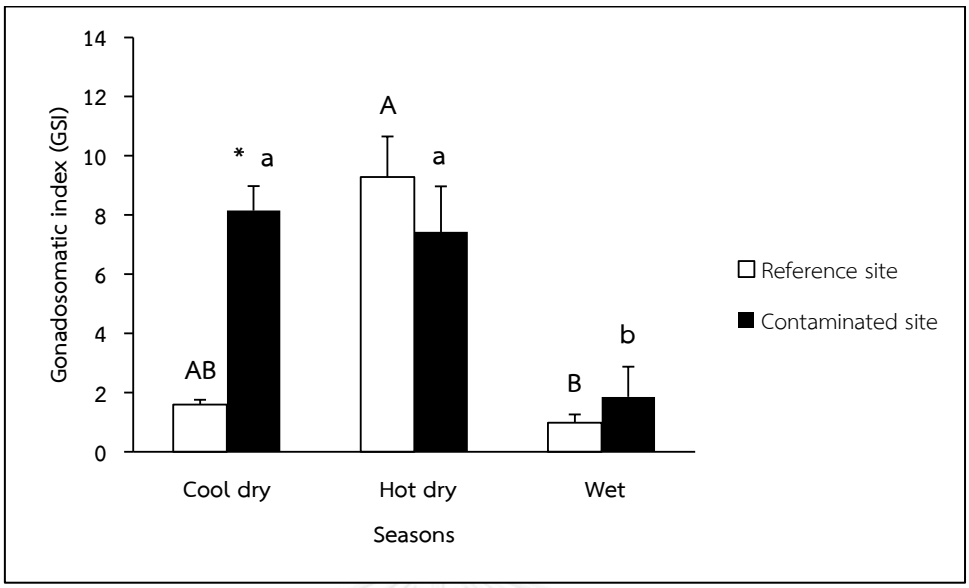


Figure 3-10 Relative ovarian weight (Mean ± S.E.M.) of female frogs *Fejervarya limnocharis* from reference and contaminated agricultural areas at Nan Province, Thailand

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Mann-Whitney Rank Sum Test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (Kruskal-Wallis One Way Analysis of Variance on Ranks, $p<0.05$; followed by Dunn's Method).

Discussions

Condition factor (CF) has been successfully used as an indicator of overall health status in this frog species in several studies (Othman et al., 2009; Othman et al., 2011; Thammachoti et al., 2012; Hegde and Krishnamurthy, 2014a; Hegde and Krishnamurthy, 2014b). In this study, different growth pattern as indicated by difference in scaling coefficient was found between these two populations of frogs living in areas with different degree of herbicide utilization. These findings are similar to previous studies on this frog (Othman, 2009; Thammachoti, 2012) and agree with several prior reports in which exposure to herbicides (especially atrazine) was found to disturb development and growth pattern of amphibians (Hayes et al., 2006; Storrs and Semlitsch, 2008; Spear et al., 2009; Lenkowski et al., 2010).

Furthermore, the difference in condition factor demonstrated that if the body length (SVL) of frogs were equal, frogs from the contaminated site would be lighter or smaller than frogs from the reference site (Thammachoti, 2012). The CF could indicate lower fitness of frogs living in the contaminated area because the smaller frogs could be easily captured as a prey in food chain (Hayes et al., 2006). Since other factors such as climate, geography, physical factors and major agricultural activities are similar between these two study sites, it seems that utilization of herbicides could affect overall health of the frogs. The findings are in line with previously published articles in which herbicides utilization was found to increase environmental stressors (Söderman et al., 2007), cause wide range of toxicities to the frog (Lajmanovich et al., 2011) or cause physiological effects on growth itself and disrupt steroidogenesis and growth hormone secretion (Hayes et al., 2006). Even if pesticides do not have immediate impacts on anuran survival, sub-lethal effects

could indirectly influence fitness and survival, and conceivably reduce population sizes over time when exposure occurs annually (Mann et al., 2009).

Significant differences of CF between periods at each site in this study were similar to the earlier study in this frog species at the same areas (Thammachoti, 2012). However, this observation was different from the studies in other frog species. In *Rana ridibunda*, it was found that condition factor of frog collected from the Northern Iran during July-September 2010 was not significantly different amoung months (Jelodar and Fazli, 2012). This could be due to a relatively shorter study period (3 months) in *R. ridibunda* compared to a year round study (every three months) in this study on *Fejervarya limnocharis*. Although, *F. limnocharis* is believed to be generalist predators that lack an apparent dietary preference, and their diets are most likely dependent on what prey is available (Norval et al., 2014), the rice frog in paddy fields could undergo aestivation when their food (insect) was decrease (Hirai and Matsui, 2001). As a result, seasonal fluctuating in condition factor could be due to different in food availability within a year.

Direct and indirect stresses of agrochemicals could affect the amphibian immune system. Spleen size is considered a useful diagnostic factor because spleen is a hematopoietic organ and its dysfunction could reflect at the whole-organism level (Anderson, 1990). However, there were only a few studies in regard to the relative spleen weight in frogs and other amphibians. In this study, the splenosomatic indices of male and female frogs were significantly different between sampling periods at both contaminated and reference sites. Significant site-related difference of splenosomatic index was also found in both male and female frogs. In other frog species, it was found that spleen mass and cellularity of spadefoot Toad

Spea spp. was different between toad in cropland versus grassland indicating that immune function may be compromised in cropland playas (McMurry et al., 2009). Previous study showed an immunosuppression of juvenile frogs (*Rana pipiens* and *Xenopus laevis*) exposed to environmentally relevant concentrations of pesticides (atrazine, metribuzin, aldicarb, endosulfane, lindane, and dieldrin). Spleen cell numbers were reduced and phagocytic activity was impaired (Christin et al., 2004).

In previous study, seasonal difference in hepatosomatic index was found in the contaminated site frogs and could be related to different level of herbicide contamination in frog tissue (Thammachoti, 2012). In this study, however, HIS of frogs from the contaminated site was relatively high throughout the year. This could indicate relatively stable exposure to xenobiotics at contaminated site where agricultural activities were found in every period (field observation). On the contrary, HIS of the reference site frogs were different between seasons with the lowest value in cool dry season when agricultural activities was minimal, and the highest value in wet season when agricultural activities were present.

Compared to previous study at the same study sites (Thammachoti et al., 2012), renosomatic index (RSI) of frog in this study showed similar trend of relatively stable levels of RSI throughout the year, indicating that kidney may be a non-target organ of herbicide exposure (Thammachoti et al., 2012). However, it is interesting to examine the underlying cause of increase RSI in reference site frogs during the wet season.

The results of gonadosomatic index of male and female frog at both sites showed a significant seasonal difference. Change in testicular weight and ovarian corresponds with reproductive activities of this frog species and in accordance with

local and seasonal patterns. In Mae Sot, Thailand, it was found that the rice frog is essentially a continuous breeder. The fluctuation in its female GSI suggested that this species is more optimized for a cyclic reproduction mode with two cycles of reproduction during the rainy season. The highest female gonadosomatic index was recorded during the months just prior to the rainy season and towards the end of the rainy season. However, the fluctuations in the male gonadosomatic index were less enhanced, suggesting that the male is sexually ready throughout the year (Othman et al., 2011).

Interestingly, GSI of both male and female frogs in contaminated site was significantly higher than those of the reference site frogs. The larger ovary in contaminated site frog, especially in cool dry season where agricultural activity was presented in this site, is possibly due to effects of herbicides on ovarian growth (Hayes et al., 2010b). Similar to previous study in this frog species, although female frogs from agricultural areas with intensive herbicide utilization had higher GSI value, the larger and heavier ovary is not always beneficial to animals. The heavier ovary with mature eggs in the contaminated site frog was found even in dry period when egg laying was not possible because temporary water bodies was dried up. Therefore, stimulating effect of atrazine herbicide may lead to reduce fecundity fitness of frog living in contaminated site in the future (Thammachoti et al., 2012).

In this study, significantly higher testicular weight in the contaminated site frogs was reported for the first time. It thus interesting to further examine whether herbicide contamination could alter the dynamics of sex hormone and resulted in increase testicular size (Wingfield and Sapolsky, 2003) or simply cause pathologic alteration of the testis (Hettyey et al., 2005; Cakici, 2013). According to Othman

(2009), testicular ovarian follicle (TOFs) was found in the testis of *F. limnocharis* caught form cadmium contaminated paddy field. The presence of TOFs could play role in increasing testicular weight of frog. Therefore, histological alteration of the rice frog testis in this study should be further examined.

Conclusion

The rice frogs were initially used as sentinels for herbicide contamination. The result of condition factor indicated that the overall health status of frog was reduced in the agricultural area where herbicide was intensively used. In addition, difference in splenosomatic index indicated that frog from both study site might exhibit different in immunological status. The markedly difference in female gonadosomatic index indicated that ovarian growth of the contaminated site frog was enhanced even in the non-breeding season (cool dry period). These results suggested that the mixed and long term use of herbicides in agricultural area could influence on health and morphology of internal organ (including immune organ) of the rice frog living in this area.

CHAPTER 4
SPECIFIC IMMUNE RESPONSES OF RICE FROG *Fejervarya limnocharis* LIVING IN AGRICULTURAL AREA AT NAN PROVINCE, THAILAND

Introduction

A current global amphibian assessment determines that amphibians are declining more rapidly than either birds or mammals (Stuart et al., 2004). There are a number of possible factors that may contribute to amphibian declines including overexploitation, habitat destruction, climate change, and disease (Stuart et al., 2004). In addition to these factors, environmental contamination of xenobiotics is believed to be capable of suppressing immune defenses and increase the effects of disease.

Since immune systems of humans and amphibians are functionally similar, the effects of a pollutant on one organism may produce similar effects in the other organism (Du Pasquier, 2000). Previous studies showed that the immune system of amphibians is as complex as that of mammals (Rollins-Smith, 2001). Amphibians are thus a candidate indicator species for monitoring effect of environmental contaminants on immune system.

Various studies have documented the effects of chemical contamination on the amphibian immune system. Many of these studies suggest that amphibians are particularly sensitive to contaminants in field observations, experimental field manipulations, and laboratory tests (Christin et al., 2003; Gilbertson et al., 2003; Christin et al., 2004; Mann et al., 2009). Experimental treatment of amphibians with common aquatic contaminants results in alterations in immune cell counts, immune

cell activities and infection intensities (Brodkin et al., 2007; Rohr and McCoy, 2010; Hayes et al., 2010a).

Since amphibians often exist in aquatic habitats that are disturbed by agricultural activities, it is possible that they are exposed to a variety of pesticides. The lymphocytes from pesticide-treated leopard frogs exhibited reduced T-cell proliferation *in vitro* in response to mitogens, and parasitism was increased in the frogs challenged with a parasitic nematode (*Rhabdias ranae*) (Christin et al., 2003). In addition the parasitic worms could migrate more rapidly to the lungs and reproduced earlier in pesticide-exposed frogs (Gendron et al., 2003). Adult Woodhouse's toads (*Bufo woodhousii*) exposed to sub-lethal doses of malathion were more susceptible to development of hepatomegaly and death due to experimental infection with the bacterium *Aeromonas hydrophila* than the control toads (Taylor et al., 1999). Specific IgM antibodies as well as oxidative burst product in whole blood were suppressed in *R. pipiens* treated with malathion, DDT, or dieldrin when compared with untreated controls.

A delayed-type hypersensitivity (DTH) response, representing the specific immune response, was enhanced in pesticide-treated frogs in comparison with control frogs. This pattern of altered immune responses observed in the laboratory was also noted in wild frogs collected in pesticide-exposed locations but not in those collected from pesticide-free locations, suggesting that the laboratory results may predict immunosuppression in natural populations (Gilbertson et al., 2003). As a result, studies on immunotoxicology of potentially immunotoxicants on amphibians, and studies of their impacts at concentrations present in the environment are reccomended.

Previous reports indicated that herbicide residues were found in both environment (atrazine) and the rice frog living in paddy fields with herbicide utilization at Nan Province (Thammachoti, 2012). Negative impacts on the frog's health were evidenced (Thammachoti et al., 2012). Therefore, it is interesting to determine the influence of herbicide utilization on the immune function of the rice frogs living in agricultural areas at Nan province.

Objective

This study aim to compare specific immune response, using delayed-type hypersensitivity (DTH) response, of the rice frog *F. limnocharis* living in agricultural areas with different degree of herbicide utilization at Wiang Sa district, Nan province.

Materials and Methods

1. Study Sites and Animal Collection

Frog sample collection was performed 4 times in January, April, July and October 2013 covering three seasons including: cool dry, hot dry and wet seasons. In each month, 40 adult rice frogs (per site) were sampled by hand after visual encounter survey (Crump and Scott, 1994) from two agricultural areas in Wiang Sa District, Nan Province (Thammachoti, 2012) including: 1) a contaminated site which is an agricultural area with intensive herbicide usage located in San Sub District (47Q 0687729 UTM 2054283) and 2) a reference site which is an organic agricultural area with no history of herbicide usage for more than 10 years located in Lai-nan Sub District (47Q 0686779 UTM 2047187). Geography, landscape and climate were similar at both study sites and herbicide utilization was the major difference between these study sites. During field sample collection, it was also found that both study sites

showed similar agricultural activities except in cool dry season (January 3013) where active agricultural activity was found only at the contaminated site (Figure 4-1).

After transportation to a laboratory at the Chulalongkorn University Forest and Research Station, Nan Province, the rice frogs from each site were individually acclimated for 2 days in plastic aquaria (Figure 4-2). During the acclimatization and immune response frogs were fed ad libitum with the small crickets and water in plastic aquaria was changed every 2 days. The rice frogs from each site were divided in to 2 groups including:

1) non-immunized frogs

Frogs were not injected with any solution during acclimatization period.

2) immunized frogs:

Frogs were immunized by injection with TiterMax® Gold Adjuvant (Sigma-Aldrich, St. Louis, USA) (Gilbertson et al., 2003) dissolved in sterile amphibian phosphate buffered saline (APBS), pH 7.5 (Robert et al., 2004; Bhattacharjee and Das, 2008). Each frog was received 100 µl of this emulsion intramuscularly at the left thigh.

Figure 4-1 Agricultural activities of two study areas in Nan Province during sampling periods including cool dry season (January 2013; A-B), hot dry season (April 2013; C-D) and wet season (July 2013; E-F and October 2013; G-H)

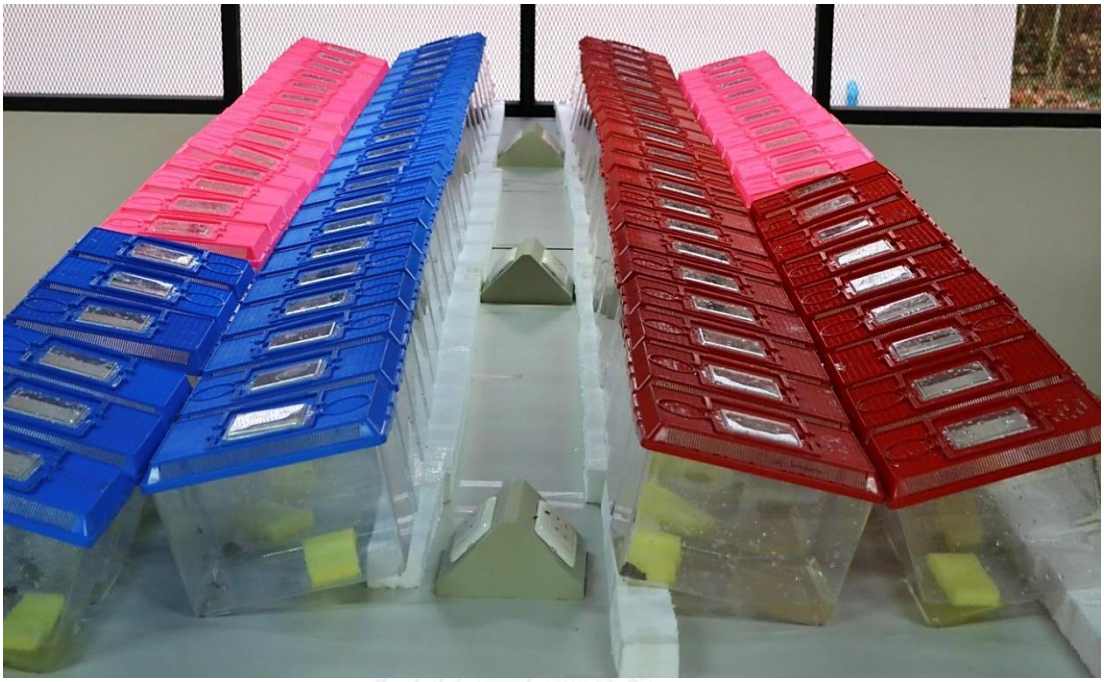


Figure 4-2 The rice frogs *Fejervarya limnocharis* were individually acclimated and raised in plastic aquaria after collected from agricultural areas at the Chulalongkorn University Forest and Research Station, Nan Province.

2. Specific Immune Response (Delayed-Type Hypersensitivity, DTH) Study

In mammals, delayed-type hypersensitivity (DTH) is known as an antigen specific cutaneous reaction mediated by helper T-cells, and usually accompanied by swelling and monocytic infiltration into the site of the lesion within 24 to 72 hrs. DTH reaction in the skin is initiated by presentation of antigen by certain antigen presenting cells to sensitized memory T-cells (Dhabhar and McEWen, 1997). DTH response could present beneficial or harmful aspects of immune function against the infection and auto immunity.

After acclimatization, the non-immunized frog (group 1) was subjected to study for specific immune response and non-specific immune response investigation. The immunized frog (Group 2) was subjected to immunization for 9 days, followed by study for specific immune response and non-specific immune response investigation (Figure 4-3).

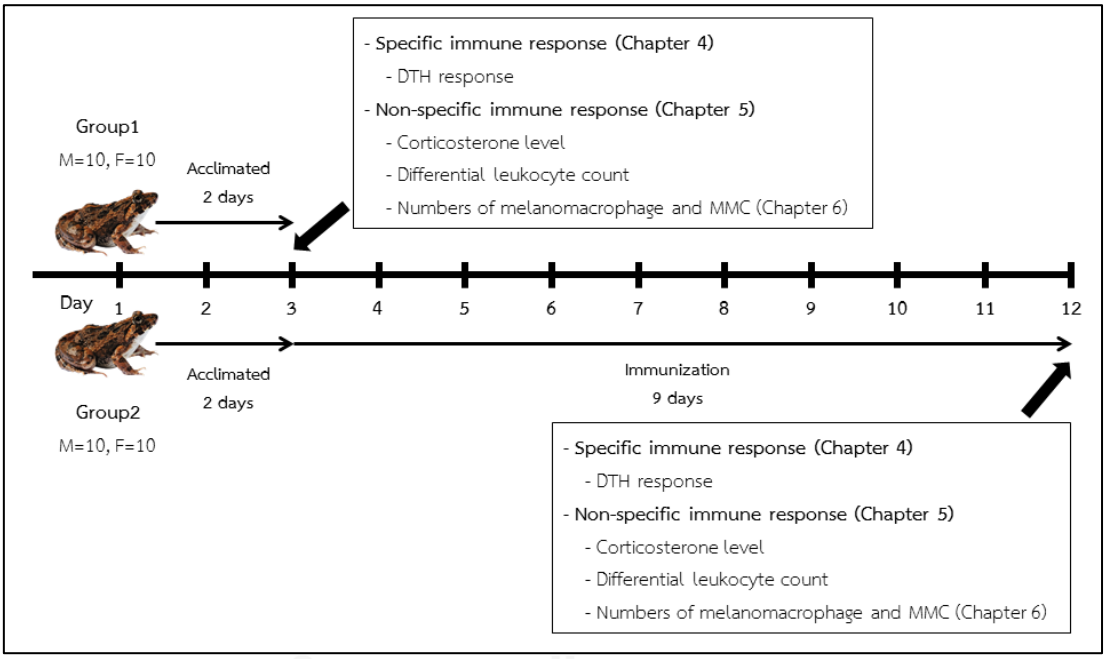


Figure 4-3 Research scheme for investigating immune response of the rice frogs caught from agricultural areas with different degree of herbicide utilization. Similar endpoints were examined in frogs from each site which were initially divided into 2 groups as followed:

Group 1: non-immunized frogs (male=10, female=10)

Group 2: TiterMax® Gold Adjuvant immunized frogs (male=10, female=10)

A common field test of T-cell mediated immunity was used in DTH response study as follows (Seiter, 2011). Using 31 Gauge needle and insulin syringe (BD Ultra-Fine™ II, New Jersey, USA), right toe at hind limb of frog was subcutaneously injected with a 20 µl of 2-mg/ml solution of phytohemagglutinin (PHA-P; Sigma-Aldrich, St. Louis, USA) in APBS, while left toe of hind limp was injected with 20 µl of sterile APBS. Thickness of toe was measured from both sides at 0, 24, 48, and 72 hrs. after injection using Moore and Wright micrometer (accuracy 0.001 mm). Differences in thickness after injection of each toe were recorded. The difference in thickness of the right toe (PHA-P injected) compared to the left toe (PBS-injected) (Gilbertson et al., 2003) was calculated and shown as the increase toe thickness (Figure 4-4).

3. Statistical Analyses

The data were tested for normal distribution (the Kolmogorov-Smirnov test) and homogeneity of variance. Mean comparison between sexes and sites were determined using Student t-test for the data that is normally distributed or Mann-Whitney Rank Sum Test for the data that is not normally distributed. Mean difference from initial measurement among seasons was determined using one way ANOVA followed by Student-Newman-Keuls Method for the data that is normally distributed or Kruskal-Wallis Analysis of Variance on Ranks for the data that is not normally distributed. All statistical analyses were performed using SigmaPlot 11.0.

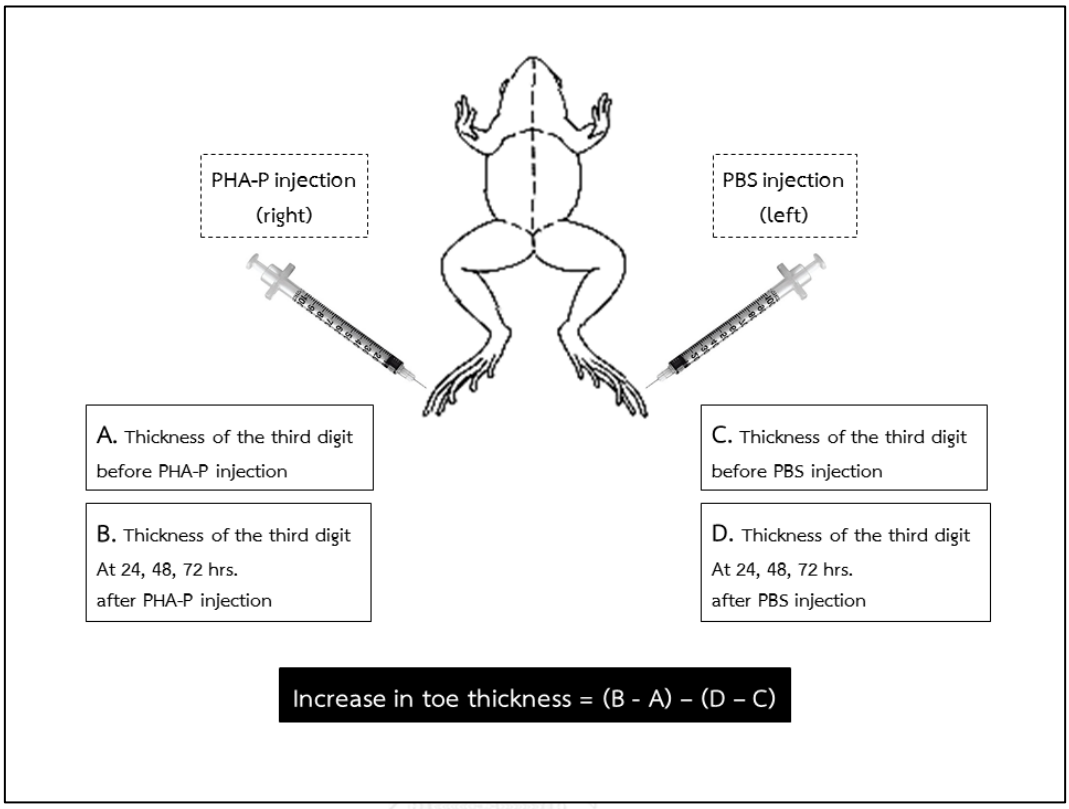


Figure 4-4 Diagram of delayed-type hypersensitivity (DTH) response study in the rice frog. Right toe at hind limb of frog was injected with phytohemagglutinin (PHA-P) in APBS, while left toe of hind limp was injected with sterile APBS. An increase in thickness was calculated by the thickness of right toe (PHA-P injection) compared to the thickness of left toe (PBS-injection).

Results

The specific immune response in term of DTH response results were reported in 3 seasons including: cold dry, hot dry and wet seasons. Specific immune response (or PHA skin response) of frogs as indicated by an average increase in toe thickness of each groups after DTH test were recorded from frogs with different sexes, immunization, sites and seasons and were illustrated in Table 4-1 - 4-2 and Figure 4-5 – 4-16. Differences in thickness between the right (PHA-injected) toe and the left (PBS-injected) toe were shown at difference interval (24, 48 and 72 hrs.). Since there was sex-related difference in DTH response ($p<0.05$; Student t-test), seasonal and site related differences were analyzed and represented separately according to sex. The common tend of DTH responses of the rice frog showed the frogs caught from the reference site have significantly higher response than frogs from the contaminated site.

1. Specific Immune Response of the Non-Immunized Frogs

The result of DTH response of non-immunized rice frogs from both contaminated and reference sites were shown in Table 4-1.

Table 4-1 Delayed-type hypersensitivity (DTH) as determined by change in toe thickness (Mean ± S.E.M.) in response to phytohemagglutinin-P (PHA-P) of the non-immunized rice frogs, *Fejervarya limnocharis*, caught from the reference site and the contaminated site at Nan Province, Thailand

Seasons	Time after injection (hrs.)	Male		Female	
		Reference site	Contaminated site	Reference site	Contaminated site
Cool dry	24	0.020 ± 0.006 [B]	0.021 ± 0.004	0.024 ± 0.010	0.050 ± 0.026
	48	0.064 ± 0.009 [*, A]	0.029 ± 0.011	0.036 ± 0.009	0.132 ± 0.049 [*]
	72	0.086 ± 0.013 [A]	0.058 ± 0.013	0.075 ± 0.014	0.146 ± 0.046
Hot dry	24	0.044 ± 0.013 [*]	-0.004 ± 0.015	0.027 ± 0.007	0.021 ± 0.008
	48	0.057 ± 0.019 [*]	0.008 ± 0.014	0.046 ± 0.012	0.028 ± 0.007
	72	0.050 ± 0.016	0.007 ± 0.014	0.055 ± 0.012 [*]	0.027 ± 0.006
Wet	24	0.038 ± 0.020	0.008 ± 0.001	0.006 ± 0.001 [B]	0.006 ± 0.001
	48	0.044 ± 0.018	0.013 ± 0.003	0.017 ± 0.003 [*, A]	0.009 ± 0.001
	72	0.044 ± 0.018 [*]	0.013 ± 0.004	0.015 ± 0.003 [A]	0.009 ± 0.002

Remarks:

- Significant site-related difference (Student t-test, $p<0.05$) is indicated by an asterisk (*). Significant difference from initial measurement (one-way ANOVA, $p<0.05$) is indicated by a letter (capital letters for reference site and small letter for contaminated site).

1.1 Male Non-Immunized Rice Frog

In cool dry season, the result of DTH response of male non-immunized rice frog showed the significant difference from initial measurement (one-way ANOVA, $p<0.05$) in the reference site population, but not in the contaminated site population. For site-related difference, DTH response of the reference site frogs was significantly higher than the contaminated site frogs at 48 hrs. after PHA-P injection (Student t-test, $p<0.05$) (Figure 4-5 I.).

In hot dry season, the result of DTH response of male non-immunized frog showed no significant difference from initial measurement (one-way ANOVA, $p>0.05$) in both reference and contaminated site populations. However, DTH response of reference site frog was significantly higher than contaminated site frogs at 24 and 48 hrs. after PHA-P injection (Student t-test, $p<0.05$) (Figure 4-5 II.).

In wet season, male non-immunized frog showed no significant difference in DTH response from initial measurement (one-way ANOVA, $p>0.05$) in both reference and contaminated site frogs. However, DTH response of the reference site frog was significantly higher than the contaminated site frog at 72 hrs. after PHA-P injection (Student t-test, $p<0.05$) (Figure 4-5 III.).

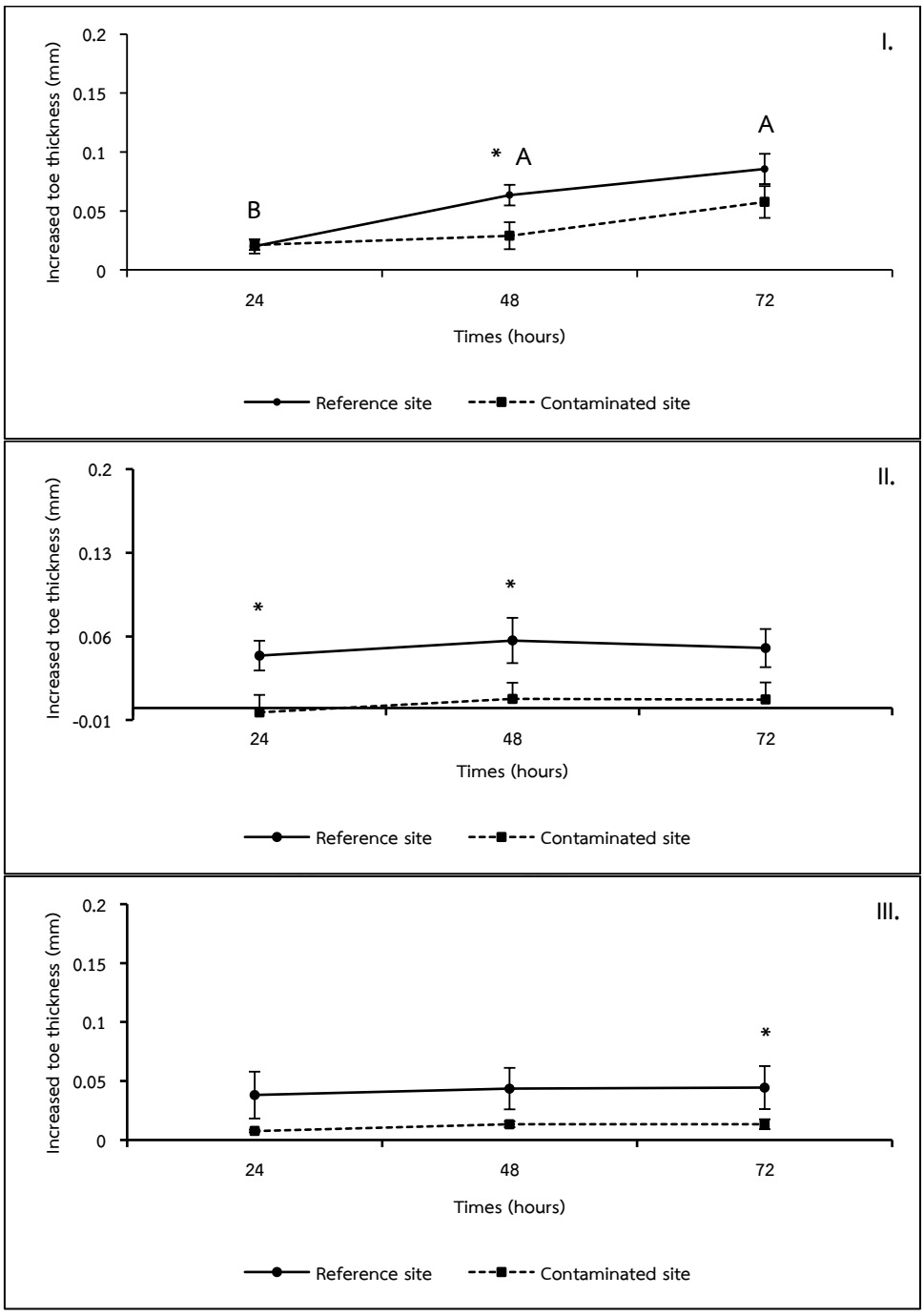


Figure 4-5 Delayed-type hypersensitivity (DTH) of the male non-immunized rice frog, *Fejervarya limnocharis*, caught from the reference site and the contaminated site in cool dry (I.), hot dry (II.) and wet season (III.) at Nan Province, Thailand

Significant site-related difference (Student t-test, $p<0.05$) is indicated by an asterisk (*). Significant difference from initial measurement (one-way ANOVA, $p<0.05$) is indicated by a letter (capital letters for reference site).

1.2 Female Non-Immunized Rice Frog

In cool dry season, there was no significant difference in DTH response of female non-immunized frogs from initial measurement (one-way ANOVA, $p>0.05$) in both reference and contaminated site populations (Figure 4-6 I.). However, DTH response of female non-immunized frog exhibited site-related difference with a significantly higher response in contaminated site frogs at 48 hrs. after PHA-P injection (Student t-test, $p<0.05$).

In hot dry season, there was no significant difference in DTH response from initial measurement (one-way ANOVA, $p>0.05$) in both reference and contaminated site populations. Nonetheless, DTH response of the reference site frog was significantly higher than the contaminated site frog at 72 hrs. after PHA-P injection (Student t-test, $p<0.05$) (Figure 4-6 II.).

In wet season, there was significant difference in DTH response from initial measurement (one-way ANOVA, $p<0.05$) in the reference site frogs, but not in the contaminated site frogs (Figure 4-6 III.). Comparison between sites revealed that DTH response of female non-immunized frog from the reference site was significantly higher than contaminated site frog at 48 hrs. after PHA-P injection (Student t-test, $p<0.05$).

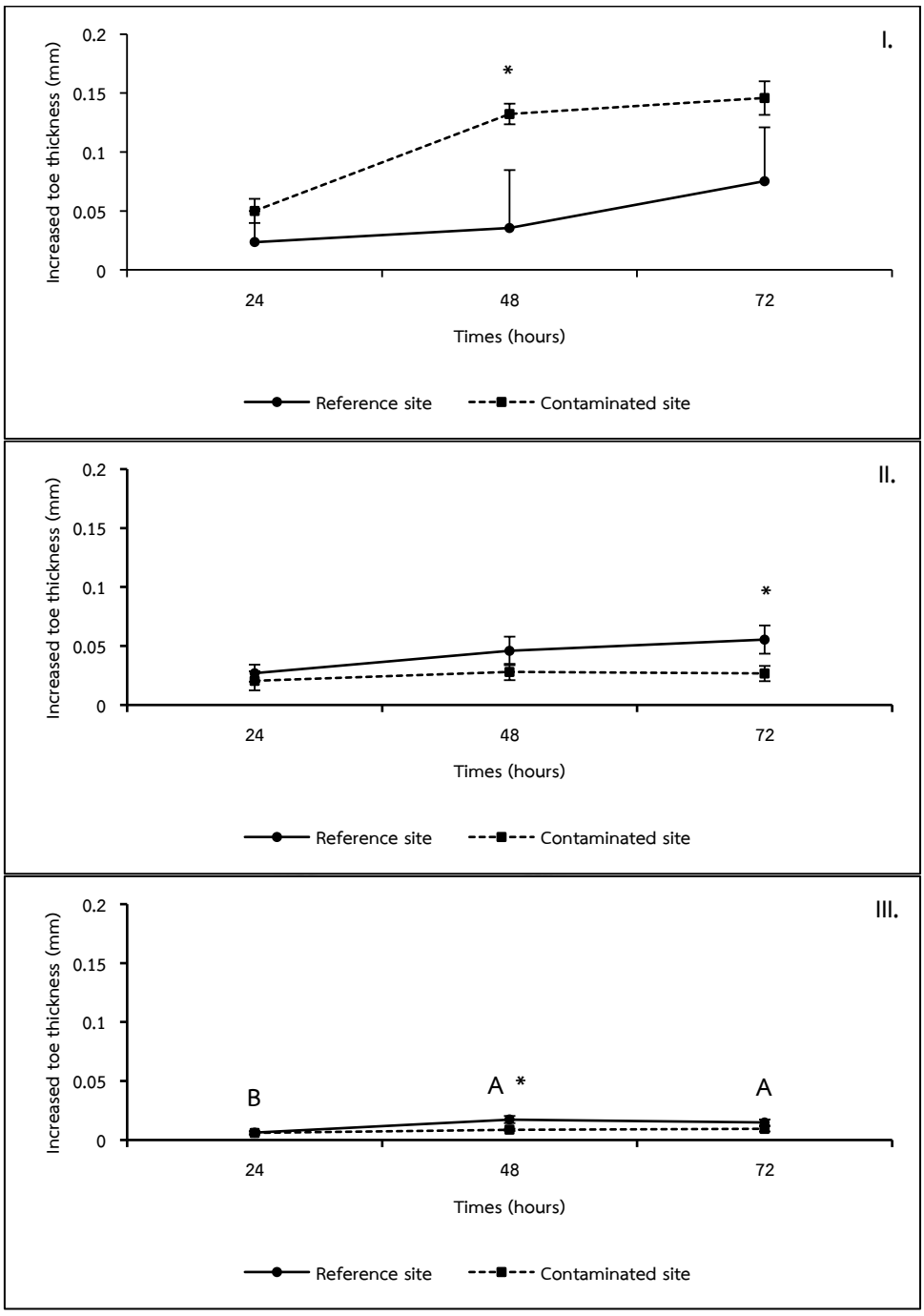


Figure 4-6 Delayed-type hypersensitivity (DTH) of the female non-immunized rice frog, *Fejervarya limnocharis*, caught from the reference site and the contaminated site in cool dry (I.), hot dry (II.) and wet season (III.) at Nan Province, Thailand
Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*). Significant difference from initial measurement (one-way ANOVA, $p<0.05$) is indicated by a letter (capital letters for reference site).

2. Specific Immune Response of the Immunized Frogs

The result of DTH response of immunized rice frogs from both contaminated and reference sites were shown in Table 4-2.

Table 4-2 Delayed-type hypersensitivity (DTH) as determined by change in toe thickness (Mean ± S.E.M.) in response to phytohemagglutinin-P (PHA-P) of the immunized rice frogs, *Fejervarya limnocharis*, caught from the reference site and the contaminated site at Nan Province, Thailand

Seasons	Time after injection (hrs.)	Male		Female	
		Reference site	Contaminated site	Reference site	Contaminated site
Cool dry	24	0.042 ± 0.016	0.025 ± 0.009	0.031 ± 0.017 ^{B}	0.031 ± 0.005
	48	0.067 ± 0.016	0.045 ± 0.010	0.065 ± 0.023 ^{B}	0.038 ± 0.004
	72	0.096 ± 0.019	0.068 ± 0.010	0.128 ± 0.030 $^{*, A}$	0.041 ± 0.004
Hot dry	24	0.020 ± 0.003	0.025 ± 0.005 ^{ab}	0.022 ± 0.003 ^{B}	0.017 ± 0.004
	48	0.032 ± 0.004	0.035 ± 0.006 ^{a}	0.051 ± 0.008 $^{*, A}$	0.023 ± 0.006
	72	0.032 ± 0.004 *	0.015 ± 0.006 ^{b}	0.052 ± 0.007 $^{*, A}$	0.021 ± 0.004
Wet	24	0.063 ± 0.035	0.017 ± 0.006	0.024 ± 0.007 ^{B}	0.019 ± 0.004
	48	0.088 ± 0.050	0.021 ± 0.007	0.041 ± 0.008 $^{*, AB}$	0.019 ± 0.003
	72	0.100 ± 0.039 *	0.019 ± 0.005	0.051 ± 0.011 ^{A}	0.213 ± 0.003

Remarks:

- Significant site-related difference (Student t-test, $p<0.05$) is indicated by an asterisk (*). Significant difference from initial measurement (one-way ANOVA, $p<0.05$) is indicated by an alphabet (capital letters for reference site and small letter for contaminated site).

2.1 Male Immunized Rice Frog

The DTH response of male immunized frog in cool dry season exhibited no significant difference from initial measurement (one-way ANOVA, $p>0.05$) in both of the reference and contaminated site population. Although the reference site frogs showed trend of higher response compared to the contaminated site frogs, there was no significant site-related difference in DTH response (Student t-test, $p>0.05$) (Figure 4-7 I.).

The result of DTH response of male immunized frog in hot dry season showed significant difference from initial measurement (one-way ANOVA, $p<0.05$) only in contaminated site population, but not in the reference site population. For site comparison, DTH response of the reference site frog was significantly greater than the contaminated site frog at 72 hrs. after PHA-P injection (Student t-test, $p<0.05$) (Figure 4-7 II.).

DTH response of male immunized frog in wet season revealed no significant difference from initial measurement (one-way ANOVA, $p>0.05$) in both reference and contaminated site frogs. However, DTH response of the reference site frog was significantly higher than the contaminated site frog at 72 hrs. after PHA-P injection (Student t-test, $p<0.05$) (Figure 4-7 III.).

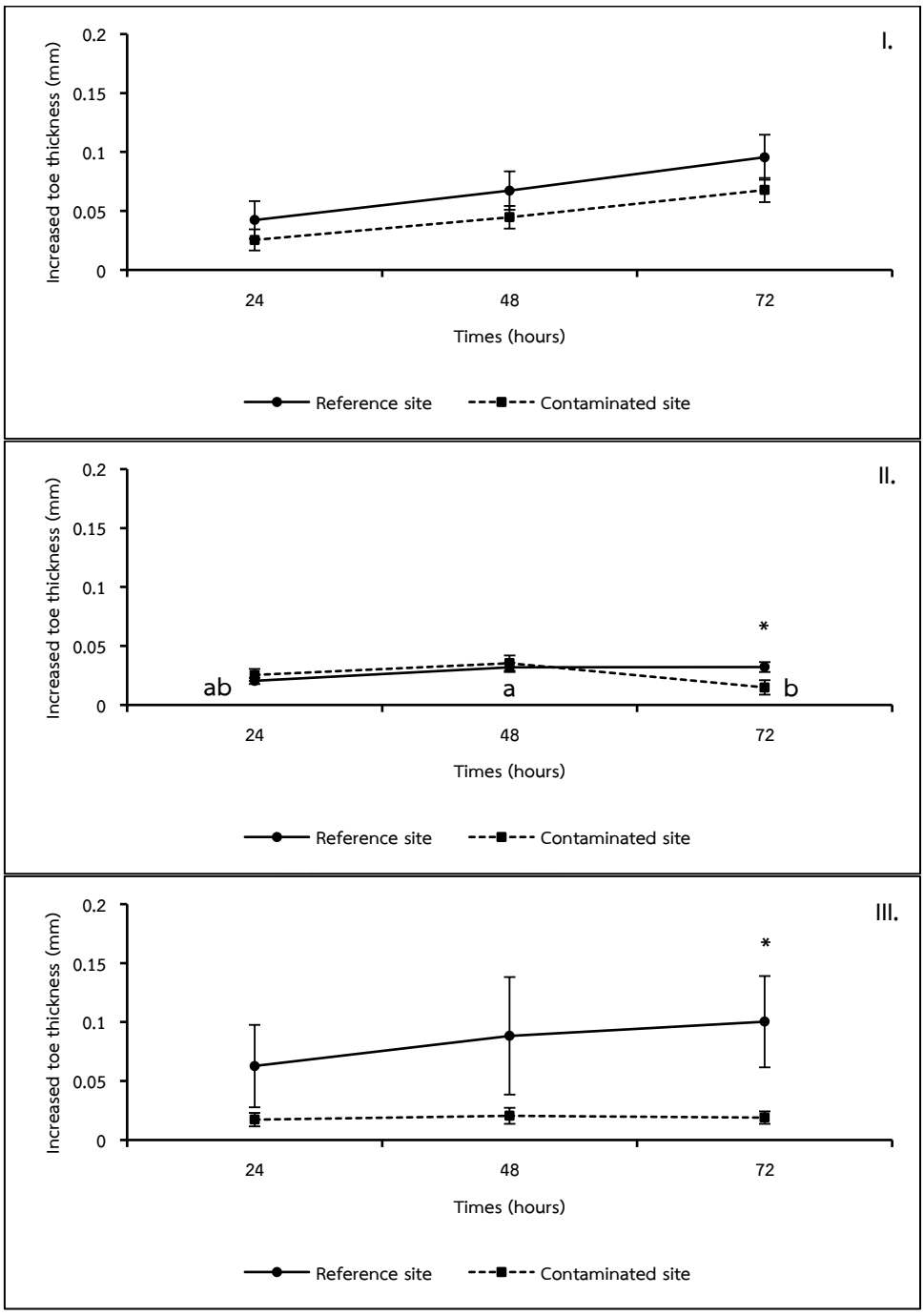


Figure 4-7 Delayed-type hypersensitivity (DTH) of the male immunized rice frog, *Fejervarya limnocharis*, caught from the reference site and the contaminated site in cool dry (I.), hot dry (II.) and wet season (III.) at Nan Province, Thailand

Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*). Significant difference from initial measurement (one-way ANOVA, $p<0.05$) is indicated by an alphabet (small letters for contaminated site).

2.2 Female Immunized Rice Frog

The DTH response of female immunized rice frog in cool dry season exhibited significant difference from initial measurement (one-way ANOVA, $p<0.05$) in the reference site frogs, but not in the contaminated site frogs. Moreover, there was significant site-related difference in DTH response with a higher response in the reference site frogs at 72 hrs. after PHA-P injection (Student t-test, $p<0.05$) (Figure 4-8 I.).

The DTH response of female immunized rice frog in hot dry season showed significant difference from initial measurement (one-way ANOVA, $p<0.05$) only in the reference site frogs. Furthermore, DTH response of the reference site frog was significantly higher than the contaminated site at 48 and 72 hrs. after PHA-P injection (Student t-test, $p<0.05$) (Figure 4-8 II.).

DTH response of female immunized rice frog in wet season showed that not only there was significant difference from initial measurement (one-way ANOVA, $p>0.05$) in reference frogs but also, DTH response of the reference site frog was significantly higher than the contaminated site at 48 and 72 hrs. after PHA-P injection (Student t-test, $p<0.05$) (Figure 4-8 III.).

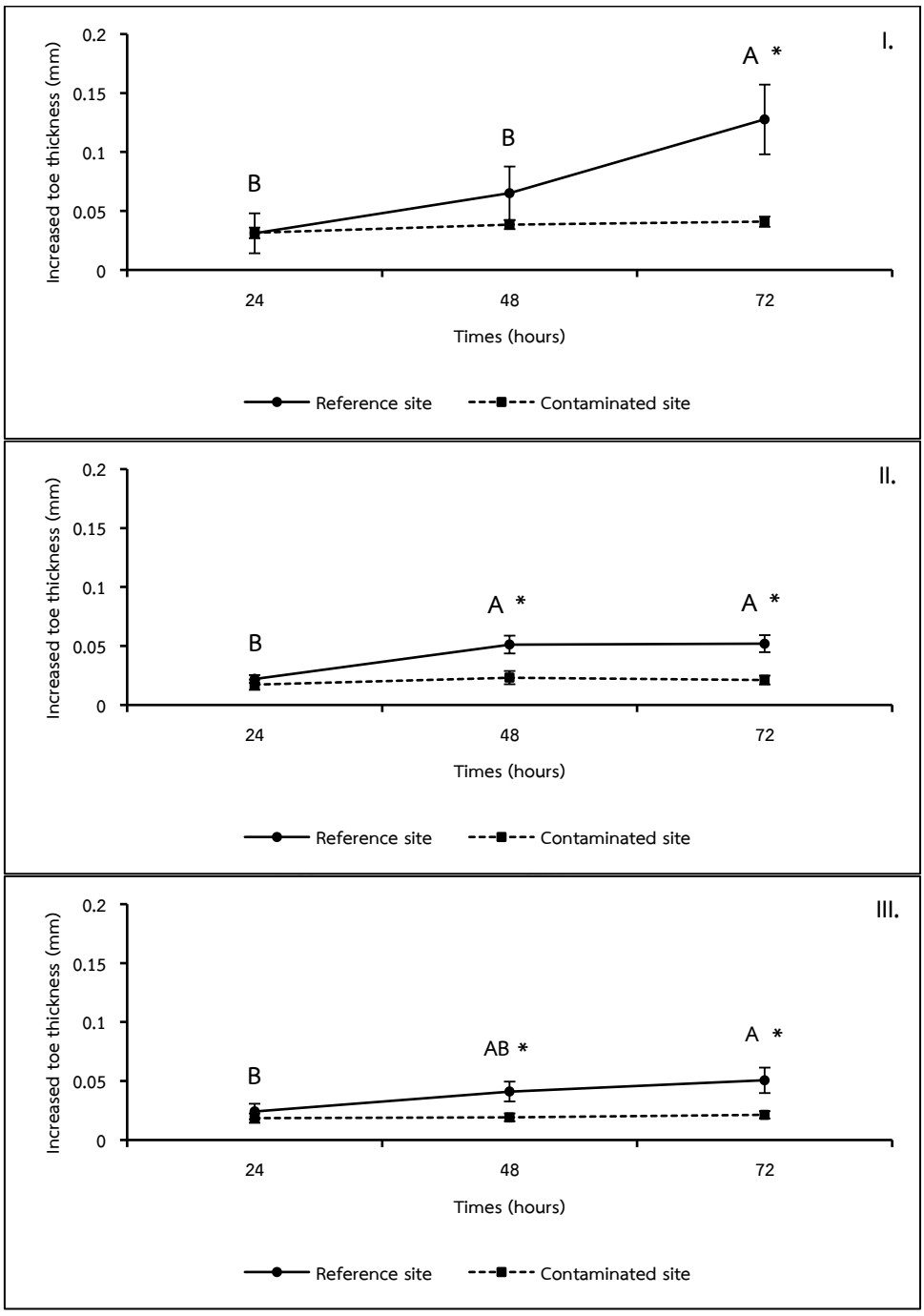


Figure 4-8 Delayed-type hypersensitivity (DTH) of the female immunized rice frog, *Fejervarya limnocharis*, caught from the reference site and the contaminated site in cool dry (I.), hot dry (II.) and wet season (III.) at Nan Province, Thailand

Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*). Significant difference from initial measurement (one-way ANOVA, $p<0.05$) is indicated by an alphabet (capital letters for reference site).

Discussions

The delayed-type hypersensitivity response (DTH response) or PHA-swelling technique has been routinely used to quantify vertebrate immune responses in various ecological situations especially in birds. In this study, the DTH responses of the rice frogs in both non-immunized and immunized stage showed the similar trend of responses with potential suppression of immune response in frogs from the contaminated site where intensively herbicide usage was found. The PHA-induced tissue swelling measurements could be used as the surrogates for an immuno-competence. However, it is still needed to be proven whether more swelling represents a better or stronger cell-mediated immune response (Martin et al., 2006).

In many organisms, PHA or a lectin derived from red kidney bean, could induces *in vitro* mitosis of T-lymphocytes which is the effector cells of the cell-mediated immune system (Naspitz and Richter, 1968). Although most pathogen-associated antigens are effective on T-lymphocytes only in the presence of cofactors and can stimulate activation of only a small proportion of T-lymphocytes, PHA can act polyclonally and induce a substantial proportion of T-lymphocytes to undergo mitosis, without cofactors. As a result, level of swelling elicited by PHA injection offer a measure of cell-mediated immunocompetence because of the mitogenic effect of PHA on T-lymphocytes. However, the reaction to PHA in some species is more complex than simple mitogenesis of T-lymphocytes. It is believed that both innate and adaptive aspects of the immune response may be initiated at the injection site (Martin et al., 2006).

Alternatively, PHA may induce an inflammatory or innate immune response as a result of being a large antigenic molecule capable of damaging tissue (Kennedy and Nager, 2006). Increase in tissue thickness following PHA injection may not be linearly related to the number of T-lymphocytes recruited to the injection site and may therefore not reflect purely cell-mediated immunity. However, innate immune responses typically are the first line of defense consisting of non-specific responses to foreign antigens or tissue damage that result in acute inflammation and rapid recruitment of granulocytes (neutrophils and eosinophils) and macrophages. Increased skin thickness at the injection site therefore could arise from edema, vascular congestion or infiltration by leucocyte types other than lymphocytes (Brown et al., 2011). Nevertheless, significant site-related difference in PHA-induced swelling found in the rice frog is a district evidence that frog from these two site showed markedly different immune function.

Interestingly, the response in female non-immunized frogs in cool dry season showed different pattern of response compare to the other groups. The higher DTH response of female frogs from the contaminated site in cool dry season could be due to an internal factor, such as reproductive cycle, of the rice frog. In cool dry season, the gonadosomatic index of female non-immunized frog from contaminated site was significantly greater compared to frog from the reference site, while there was no site-related difference in GSI of the male non-immunized frogs (Chapter 3). Since herbicide used in agricultural area of Nan Province, especially atrazine, is a known endocrine-disrupting chemical (Kavlock, 2001; McKinlay et al., 2008) that could exert several estrogenic effects (Hayes et al., 2002; Hayes et al., 2006; Kloas

and Lutz, 2006; Hayes et al., 2010b). It might be possible that the unnaturally larger ovary of the contaminated site frog is due to effects of herbicides on ovarian growth.

One of the explanations for the link between the immune and reproductive systems might relate to resource partitioning. Under this hypothesis, maintaining a competent immune system would incur an energetic cost. Thus, the resources required to preserve a functional system and to mount specific immune responses may be drawn away from other key physiological processes, such as growth and reproduction (Sheldon and Verhulst, 1996; Norris and Evans, 2000). In a high-risk disease scenario, devoting resources for reproduction might decrease future reproductive success through the effects of low investment in immune responses (Gustafsson et al., 1994). The trade-off between key physiological processes works in both directions. During times when energetic demands are high (e.g. during reproduction) immune function may be decreased to allow an individual to maximize its reproductive effort, thereby increasing the likelihood of successful survival of offspring (Norris and Evans, 2000) with the potentially increasing susceptibility to infection.

Difference pattern of response in female non-immunized frogs in cool dry season might be related to difference in agricultural activity between reference and contaminated site in cool dry season (Figure 4-1 A-B). In cool dry season, no agricultural activity was found in reference site but there was corn and tobacco cultivation in the contaminated site. It was possible that herbicides were used in this area during cool dry season. Therefore, frogs may be exposed to other immunosuppressive contaminants other than pesticides. It is also possible that frogs living in this agricultural area are under stressful conditions that could affect the frog

immune system (Rollins-Smith, 2001). Therefore, human activities around the contaminated site might be partly responsible for the difference in the DTH response observed in these frogs.

In this study, the DTH responses of the rice frog showed that frogs caught from the reference site tended to have higher response than frogs from the contaminated site. Similar to a study in the cane toad (*Rhinella marina*), the test was found to be reliable in quantifying immune response in PHA skin-swelling assay (Brown et al., 2011). However, previous study in other frog species indicated that there was a slightly greater response in *Litoria infrafrenata* compared with *L. caerulea*. The DTH response in either of the two *Litoria* species was minimal and variable in contrast to other taxa and was not found to be a sensitive indicator of immune function (Young et al., 2014). The DTH response observation in adult *R. pipiens* was also found to be highly variable and less sensitive for detecting pesticide-related immune suppression compared with hemocyanin-specific antibody and whole blood chemiluminescence tests. As a result, it may be possible that the exact effect of contaminants on the DTH reaction, suppression or enhancement, might be species specific (Gilbertson et al., 2003).

Conclusion

Previous reports showed that herbicide contaminations were found in environment and the tissue of the rice frog, *F. limnocharis*, living in paddy fields at Nan Province and negative impacts on the frog's health were evidenced. In this study, the DTH response was used as the marker of specific immune response in the rice frog. In general, frogs caught from the reference site tended to have higher response than frogs from the contaminated site. Although, there are species different in the PHA skin-swelling, this result suggested that the PHA method could provide a robust measure of anuran immune response. These results suggested that the mixed and long term use of herbicides in agricultural area at Nan Province could suppress the immune function of the rice frog inhabiting in this area.

CHAPTER 5
NON-SPECIFIC IMMUNE RESPONSES OF RICE FROG *Fejervarya limnocharis* LIVING IN AGRICULTURAL AREAS AT NAN PROVINCE, THAILAND

Introduction

Environmental contamination may have both lethal and sublethal effects on natural population of vertebrates. Amphibians are vulnerable to a variety of contaminants, including pesticides, heavy metals, and other pollutants which could be arisen from various urban, industrial, and agricultural sources (Shutler and Marcogliese, 2011). In addition, amphibian declines have also been implicated by the natural stressors, such as parasites and pathogens (Stuart et al., 2004; Vredenburg et al., 2010). Immune responses of amphibian could be weaken by contaminants (Carey et al., 1999; Voccia et al., 1999), potentially leading to increased risk of parasitism and disease, and endangering their populations. In particular, amphibians that are exposed to environmental stressor such as pesticides applied during aquatic larval phase of development or adults phase in wetlands ecosystem may showed sign of response followed by deficits in immune function (Carey et al., 1999).

Exposure of vertebrates to a wide variety of different stressors (e.g. xenobiotic substance) could activate the hypothalamus-pituitary-adrenal (HPA) axis and elevates plasma levels of the glucocorticoid hormone (Carr, 2011). In amphibian, corticosterone is the primary glucocorticoid hormone secreted by the interrenal glands (Jungreis et al., 1970). The role of glucocorticoid hormones in immune regulation in vertebrates is well established (Sapolsky et al., 2000). Short–term

studies of increased corticosterone in amphibians have provided valuable information on endocrine influence of immunity (Bennett et al., 1972; Tournefier, 1982; Garrido et al., 1987).

There are recent evidences that xenobiotic chemicals in the environment can induce increase in circulating corticosteroids (Gendron et al., 1997; Hopkins et al., 1999). The elevated resting levels of corticosterone and decreased responsiveness to ACTH were shown in toads (*Bufo terrestris*) collected in areas polluted by coal ash (Hopkins et al., 1999) or mudpuppies (*Necturus maculosus*) exposed to organochlorines (Gendron et al., 1997). The maximal concentrations of corticosteroid level were recorded in association with immunosuppression at metamorphosis (Rollins-Smith and Blair, 1993; Barker et al., 1997; Rollins-Smith et al., 1997). It is possible that environmental factors may indirectly suppress immune defenses through elevation of corticosterone, resulting in increased susceptibility to disease as seen in an increases in the susceptibility of malathion-exposed Woodhouse's toads (*Bufo woodhousi*) to *Aeromonas hydrophila* (Taylor et al., 1999).

Glucocorticoids (corticosterone) level has many applications in ecology and physiology. However, there are drawbacks associated with it. For example, levels of plasma corticosterone rise quickly immediately following capture of wild animals (Romero and Reed, 2005), thus making it difficult to obtain baseline measurements in field situations. The use of hematological parameters such as relative white blood cell (WBC) counts made from blood smears has been suggested as an alternative method for measuring corticosterone. Since, increase in corticosterone cause changes in the leukocyte component of the vertebrate immune system that can be quantified and related to hormone levels. Moreover, the leukocyte approach offers

certain advantages over direct corticosterone measurement that it does not require rapid sampling, its cost is relatively inexpensive and its response to stress is conserved across taxonomic groups (Davis et al., 2008).

Vertebrate immune function could be monitored by measuring leukocyte ratios (Norris and Evans, 2000). Most vertebrates have five types of white blood cells (WBCs): including lymphocytes, neutrophils, eosinophils, basophils and monocytes. Neutrophils and monocytes are phagocytic and generally associated with investment in innate immunity, whereas lymphocytes, the smallest leukocyte, represent investment in acquired immunity. Eosinophils are cytotoxic cells that stimulate other WBC to release histamines and protect hosts from helminth parasites (Edwards, 1994). Monocytes are long-lived phagocytic cells associated with defence against infections and bacteria (Campbell, 1995; Davis et al., 2004). For basophils, their function is not clearly understood (Rupley, 1997) but possibly involves with inflammation (Campbell, 1995). In birds and mammals, higher heterophil (referred to as neutrophil in amphibians) to lymphocyte ratios could indicate various kinds of stressor, including fasting, foreign antigens, pesticides, handling, blood-sampling, and injury (Gross and Siegel, 1983; Vleck et al., 2000; Work et al., 2001; Shutler et al., 2004). These reports suggested that neutrophil: lymphocyte ratios in amphibians (Forbes et al., 2006; Davis et al., 2008), could be considered as a general response to stressor.

Environmental stress (herbicides) found in both environment (atrazine) and the rice frog living in paddy fields with herbicide utilization at Nan Province (Thammachoti, 2012) can increase stress hormones level and subsequently change in the leukocyte profile of vertebrates. In this study, therefore, it is interesting to

determine the influence of herbicide utilization on the corticosterone level and leukocyte profile (N: L ratio) associated with non-specific immune response of the rice frogs living in agricultural areas at Nan province.

Objective

To compare non-specific immune response (corticosterone level and neutrophil: lymphocyte ratio) of the rice frog *F. limnocharis* living in agricultural areas with different degree of herbicide utilization at Wiang Sa District, Nan Province.

Materials and Methods

1. Animal Collection and Experiment

The rice frogs from reference site and contaminated site were surveyed and sampled as previous described (Chapter 4). These frogs were acclimatized and divided into 2 groups including:

1) non-immunized frogs

Frogs were not injected with any solution during acclimatization.

2) immunized frogs:

Frogs were immunized by injection with TiterMax® Gold Adjuvant dissolved in sterile amphibian phosphate buffered saline (APBS), pH 7.5. Each frog was injected with 100 µl of this emulsion intramuscularly at the left thigh.

After acclimatization and specific immune response investigation (see Chapter 4), the non-immunized frogs (group 1) were subjected to study for non-

specific immune response. The immunized frogs (group 2) were subjected to immunization for 9 days after acclimatization, followed by study for specific and non-specific immune response investigation (Figure 4-3).

2. Non-Specific Immune Response studies

2.1 Corticosterone Level

After euthanasia, blood was collected by cardiac puncture technique using 27 G insulin syringes, and plasma was collected at -20°C after centrifuged. Subsequently, corticosterone level was determined using enzyme-linked immunosorbent assay (ELISA) technique.

Corticosterone in plasma of rice frog samples was measure by the DetectX® corticosterone immunoassay kit. Frog plasma was mixed with dissociation reagent at the ratio of 1:1 before diluted (1:25) with assay buffer. Standard corticosterone were prepared from stock solution in order to get a serially diluted standard at concentration of 10,000 to 78.125 pg/mL. Duplicate samples of standards and diluted plasma (50 µl each) were pipetted into a clear microtiter plate coated with an antibody to capture sheep antibodies. Seventy five microliters of assay buffer were added in duplicate into each well to act as non-specific binding (NSB) and 50 µl of assay buffer was added as duplicate into the plate and used as maximum binding (B0 or 0 pg/mL). A corticosterone-peroxidase conjugate (25 µl) was added to every wells of microplate. The competitive binding reaction was initiated by an addition of a 25 µL polyclonal antibody to corticosterone to each well (except NSB wells). After an hour incubation, each well of the plate the plate was washed with 300 µL wash buffer for 4 times. Subsequently, 100 µL of TMB substrate was added in to each well so that the substrate could react with the bound

corticosterone-peroxidase conjugate for 30 minutes. The reaction was stopped by an addition of 50 µL of the stop solution to each well. The intensity of the generated color was detected in a microtiter plate reader capable of measuring 450 nm wavelength (Multiskan EX).

To calculate for corticosterone concentration, the following steps were employed. Average value of absorbance at 450 nm of NSB wells were used to subtract from the value of each standards and samples. Then, the net absorbance of each standard/ sample was divided by net absorbance of the maximum binding (B0) and multiplied by 100. Plots of corticosterone concentrations and %B/ B0 of standards as well as linear regression line were generated and used as a standard %B/B0 curve (Figure 5-1). The sample concentrations were obtained and calculated from the standard %B/B0 curve. The concentration of e corticosterone in the sample was calculated, after making suitable correction for the dilution of the sample.

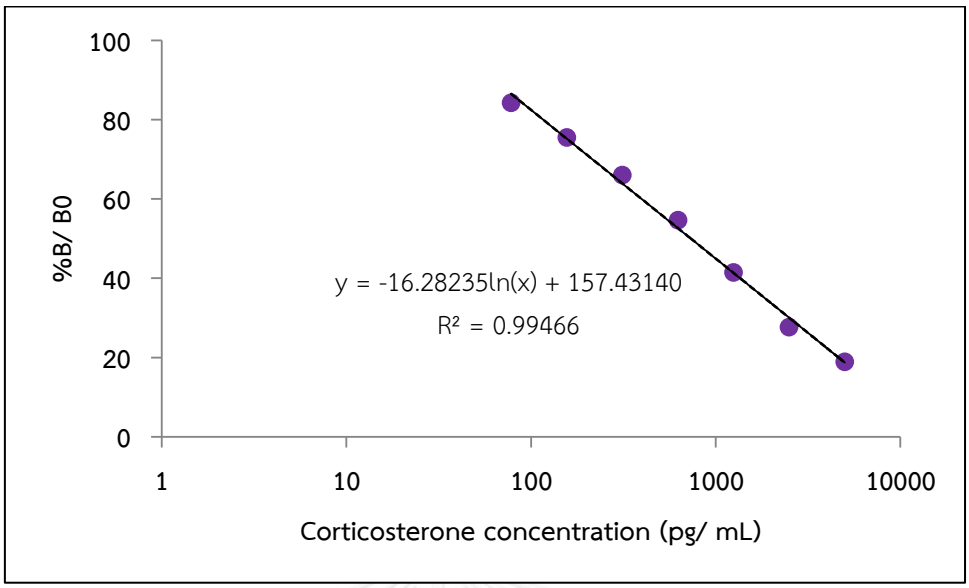


Figure 5-1 Standard curve between corticosterone concentration and %B/ B0 for calculation of the plasma corticosterone level.

In this study, coefficient of determination (R^2) of the standard curves ranged from 0.9599 to 0.9947, and the coefficient of variation for inter assay variation was 18.22%.

2.2 Differential Leukocyte Counts (Neutrophil: Lymphocyte Ratio)

Since elevated level of corticosterone could increase number of neutrophils and decreases in lymphocyte numbers, and the relative proportion of neutrophils to lymphocytes (or N: L ratio) is positively related to the environmental stressor (reviewed by Davis et al. (2008)), differential leukocyte count was performed with frog blood sample and used as marker of non-specific immune response.

Blood of rice frog was smeared on a cleaned glass slide, air dried, fixed with methanol and stained with Giemsa stain. Leukocytes were classified into neutrophil, eosinophil, basophil, monocyte and lymphocyte (Davis and Durso, 2009)

and counted under light microscope (Olympus CH-2). Then, ratio of neutrophils: lymphocytes was calculate (Davis et al., 2008).

3. Statistical Analyses

The data were tested for normal distribution (the Kolmogorov-Smirnov test) and homogeneity of variance. Mean comparison between sexes and sites were determined using Student t-test for the data that is normally distributed or Mann-Whitney Rank Sum Test for the data that is not normally distributed. Mean difference between seasons was determined by one way ANOVA followed by Student-Newman-Keuls Method (normally distributed data) or Kruskal-Wallis Analysis of Variance on Ranks (non-normally distributed data). All statistical analyses were performed using SigmaPlot 11.0.

Results

1. Corticosterone Level

There was a significant difference in plasma corticosterone of male and female frog (Student t-test or Mann-Whitney rank sum test, $p<0.05$). Therefore the data between male and female was analyzed separately (Figure 5-2 – 5-5).

For the non-immunized frogs, no significant seasonal- and site-related difference in plasma corticosterone was found in both male (Table 5-1) and female frogs (Table 5-2) (one-way ANOVA, $p>0.05$). However, the difference in corticosterone level was found in frogs that were injected with TiterMax® Gold Adjuvant so that their immune would be enhanced. The male immunized frogs caught from contaminated site showed significantly higher level of corticosterone compared to frog from the reference site in wet season (Student t-test, $p<0.05$; Table 5-3). Moreover, seasonal difference in corticosterone level was found in male immunized frog caught from reference site (one-way ANOVA, $p<0.05$). In female immunized frogs, there was no seasonal difference in corticosterone level (one-way ANOVA, $p>0.05$) but frogs caught from the reference site showed significantly higher level of corticosterone compared to frog from the contaminated site in cool dry and hot dry seasons (Student t-test, $p<0.05$; Table 5-4).

Table 5-1 Plasma corticosterone level (ng/mL) (Mean ± S.E.M.) of male non-immunized *Fejervarya limnocharis* populations caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Plasma corticosterone level (ng/mL)	
		Contaminated site	Reference site
Cool dry	Jan 2013	32.00 ± 4.93 (N=9)	29.20 ± 6.35 (N=13)
Hot dry	April 2013	16.43 ± 4.64 (N=12)	23.89 ± 6.81 (N=11)
Wet	Jul–Oct 2013	42.81 ± 10.39 (N=14)	53.13 ± 14.06 (N=9)

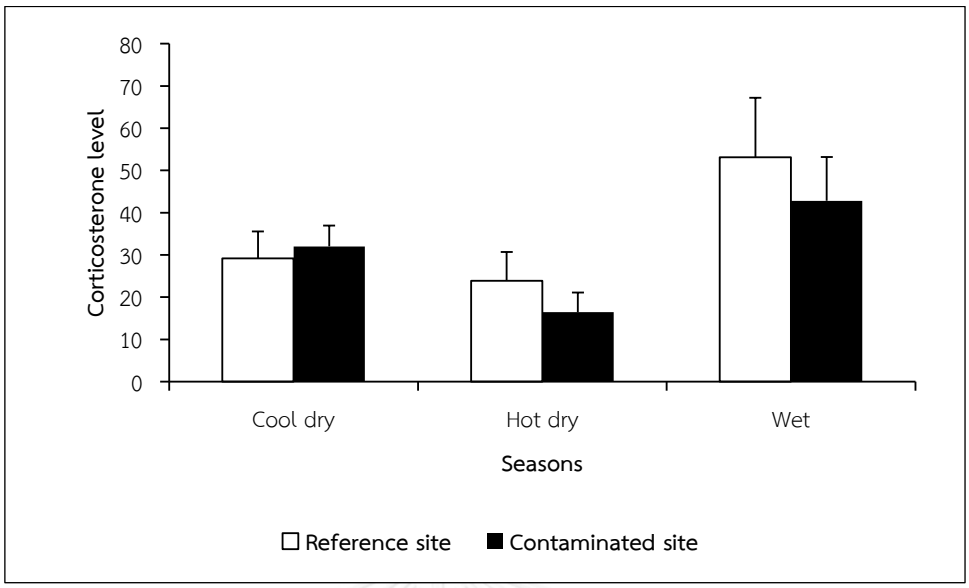


Figure 5-2 Plasma corticosterone levels (ng/mL) of male non-immunized *Fejervarya limnocharis* from reference and contaminated sites at Nan Province, Thailand

Table 5-2 Plasma corticosterone level (ng/mL) (Mean ± S.E.M.) of female non-immunized *Fejervarya limnocharis* populations caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Plasma corticosterone level (ng/mL)	
		Contaminated site	Reference site
Cool dry	Jan 2013	32.77 ± 9.90 (N=9)	38.13 ± 9.10 (N=7)
Hot dry	April 2013	23.20 ± 6.39 (N=10)	19.41 ± 2.19 (N=11)
Wet	Jul–Oct 2013	40.18 ± 6.38 (N=14)	46.73 ± 13.36 (N=16)

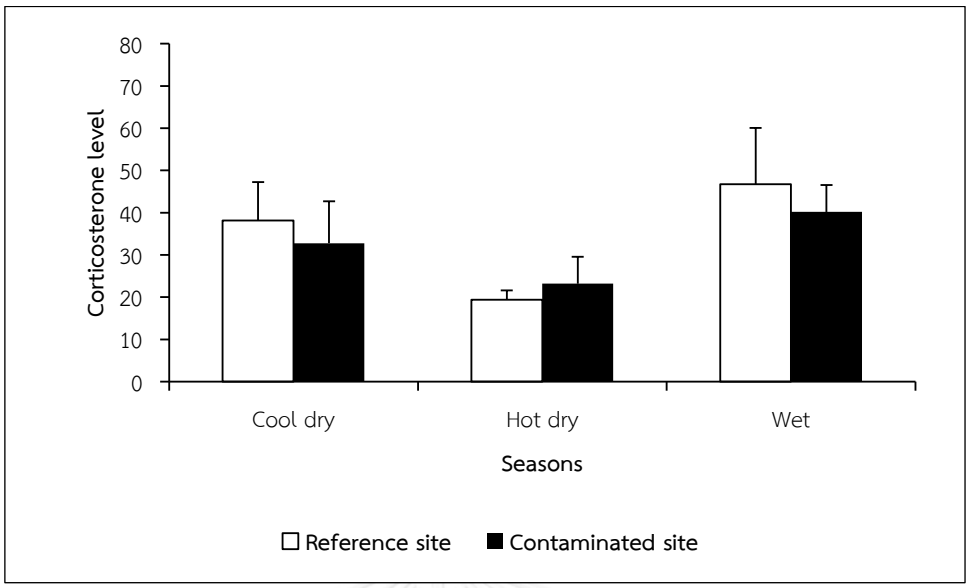


Figure 5-3 Plasma corticosterone level (ng/mL) of female non-immunized *Fejervarya limnocharis* from reference and contaminated sites at Nan Province, Thailand

Table 5-3 Plasma corticosterone level (ng/mL) (Mean ± S.E.M.) of male immunized *Fejervarya limnocharis* populations caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Plasma corticosterone level (ng/mL)	
		Contaminated site	Reference site
Cool dry	Jan 2013	19.68 ± 3.71 [b] (N=9)	18.06 ± 4.31 (N=8)
Hot dry	April 2013	31.30 ± 4.82 [b] (N=10)	37.18 ± 10.23 (N=10)
Wet	Jul–Oct 2013	54.60 ± 14.81 [*, a] (N=5)	28.14 ± 12.73 (N=4)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

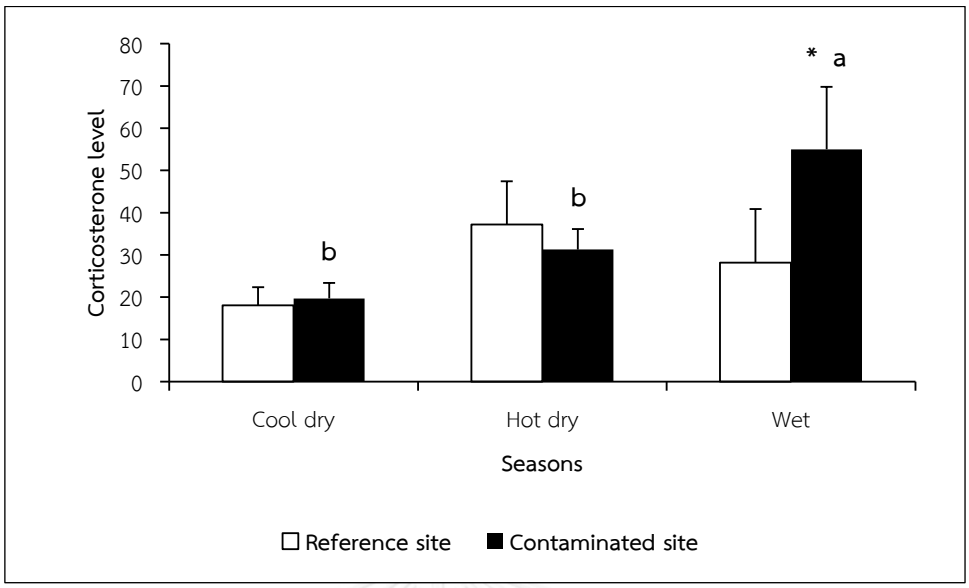


Figure 5-4 Plasma corticosterone level (ng/mL) of male immunized *Fejervarya limnocharis* from reference and contaminated sites at Nan Province, Thailand

Remarks:

- Significant site-related difference (Student t-test, $p<0.05$) is indicated by an asterisk (*).

- Significant seasonal difference (one-way ANOVA, $p<0.05$) is indicated by different letter.

Table 5-4 Plasma corticosterone level (ng/mL) (Mean ± S.E.M.) of female immunized *Fejervarya limnocharis* populations caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Plasma corticosterone level (ng/mL)	
		Contaminated site	Reference site
Cool dry	Jan 2013	21.95 ± 3.48 (N=11)	34.18 ± 5.78 * (N=10)
Hot dry	April 2013	21.14 ± 4.72 (N=12)	43.40 ± 8.67 * (N=9)
Wet	Jul–Oct 2013	48.95 ± 10.92 (N=11)	41.59 ± 9.59 (N=11)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).

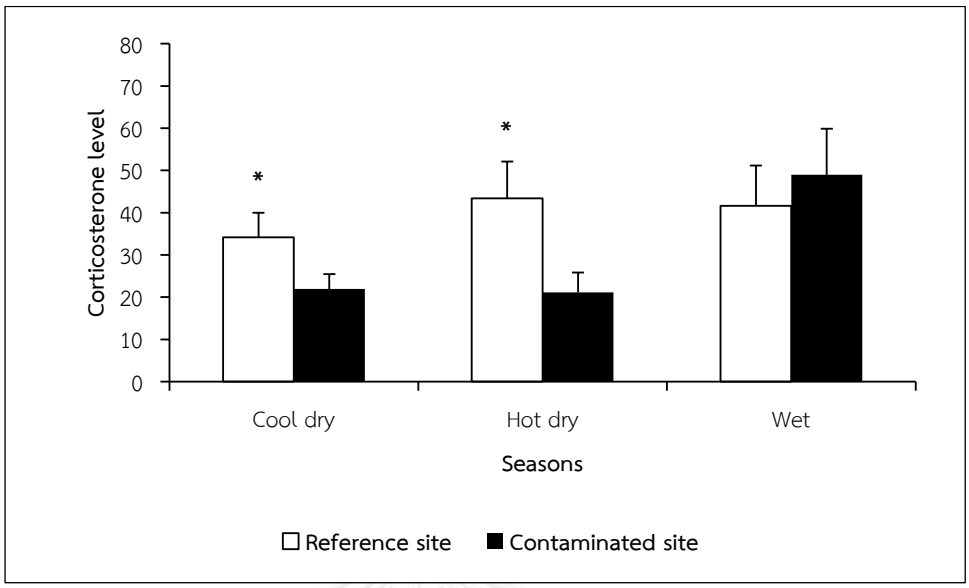


Figure 5-5 Plasma corticosterone level (ng/mL) of female immunized frogs *Fejervarya limnocharis* from reference and contaminated sites at Nan Province, Thailand

Remarks:

- Significant site-related difference (Student t-test, $p<0.05$) is indicated by an asterisk (*).

2. Differential Leukocyte Count (Neutrophil: Lymphocyte ratio)

Leukocytes of the rice frog were classified into neutrophil, eosinophil, basophil, monocyte and lymphocyte. Afterward, 100 leukocytes were counted (differential count; Table 5-5 – 5-8). Significant site-related difference was found only in the cool dry season. In general, frogs caught from the contaminated site showed a significantly lower neutrophil number and a significantly higher lymphocyte number compared to those of the reference site frogs. A specific case of decreased monocyte number in the contaminated site frog was also found with the female immunized frogs in cool dry season.

Table 5-5 Differential leukocyte count (Mean ± S.E.M.) of male non-immunized *Fejervarya limnocharis* populations caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Differential leukocyte count (%)									
		Neutrophil		Eosinophil		Basophil		Monocyte		Lymphocyte	
		Con.	Ref.	Con.	Ref.	Con.	Ref.	Con.	Ref.	Con.	Ref.
Cool dry	Jan 2013	12.00 ± 2.52 (N=3)	46.00 ± 5.77 * (N=3)	12.67 ± 1.86 (N=3)	12.33 ± 5.33 (N=3)	0.00 ± 0.00 (N=3)	0.00 ± 0.00 (N=3)	3.00 ± 1.73 (N=3)	9.67 ± 2.19 (N=3)	72.33 ± 1.20 (N=3)	32.00 ± 4.04 * (N=3)
Hot dry	April 2013	11.00 ± 1.73 (N=3)	10.00 ± 1.73 (N=3)	4.00 ± 3.46 (N=3)	2.33 ± 1.86 (N=3)	0.00 ± 0.00 (N=3)	0.67 ± 0.33 (N=3)	0.33 ± 0.33 (N=3)	0.67 ± 0.67 (N=3)	84.67 ± 1.45 (N=3)	86.33 ± 1.45 (N=3)
Wet	Jul–Oct 2013	21.50 ± 5.86 (N=6)	23.83 ± 5.10 (N=6)	2.33 ± 1.05 (N=6)	2.16 ± 0.48 (N=6)	0.00 ± 0.00 (N=6)	0.00 ± 0.00 (N=6)	1.83 ± 0.56 (N=6)	2.00 ± 0.58 (N=6)	74.33 ± 6.38 (N=6)	72.00 ± 5.27 (N=6)

Remarks:

- Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*) and solid outline around the data.

Table 5-6 Differential leukocyte count (Mean ± S.E.M.) of female non-immunized *Fejervarya limnocharis* populations caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Differential leukocyte count (%)									
		Neutrophil		Eosinophil		Basophil		Monocyte		Lymphocyte	
		Con.	Ref.	Con.	Ref.	Con.	Ref.	Con.	Ref.	Con.	Ref.
Cool dry	Jan 2013	22.33 ± 8.29 (N=3)	51.67 ± 2.33 * (N=3)	2.67 ± 0.88 (N=3)	11.33 ± 5.49 (N=3)	0.33 ± 0.33 (N=3)	0.00 ± 0.00 (N=3)	3.67 ± 2.73 (N=3)	7.00 ± 4.16 (N=3)	71.00 ± 10.07 (N=3)	30.00 ± 2.00 * (N=3)
Hot dry	April 2013	13.67 ± 2.85 (N=3)	14.00 ± 7.09 (N=3)	2.00 ± 0.58 (N=3)	5.67 ± 1.86 (N=3)	0.00 ± 0.00 (N=3)	0.00 ± 0.00 (N=3)	0.67 ± 0.33 (N=3)	0.00 ± 0.00 (N=3)	83.67 ± 3.28 (N=3)	80.33 ± 8.17 (N=3)
Wet	Jul–Oct 2013	22.00 ± 9.95 (N=6)	12.12 ± 6.06 (N=4)	0.83 ± 0.48 (N=6)	1.00 ± 0.71 (N=4)	0.00 ± 0.00 (N=6)	0.00 ± 0.00 (N=4)	1.17 ± 1.17 (N=6)	1.25 ± 0.63 (N=4)	76.00 ± 10.12 (N=6)	83.25 ± 6.10 (N=4)

Remarks:

- Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*) and solid outline around the data.

Table 5-7 Differential leukocyte count (Mean ± S.E.M.) of male immunized rice frog *Fejervarya limnocharis* population caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Differential leukocyte count (%)									
		Neutrophil		Eosinophil		Basophil		Monocyte		Lymphocyte	
		Con.	Ref.	Con.	Ref.	Con.	Ref.	Con.	Ref.	Con.	Ref.
Cool dry	Jan 2013	27.00 ± 3.79 (N=3)	55.67 ± 2.60* (N=3)	18.33 ± 5.81 (N=3)	7.33 ± 4.06 (N=3)	0.00 ± 0.00 (N=3)	0.00 ± 0.00 (N=3)	7.00 ± 4.04 (N=3)	11.33 ± 1.86 (N=3)	47.67 ± 12.35 (N=3)	25.67 ± 2.03 (N=3)
Hot dry	April 2013	11.33 ± 2.03 (N=3)	11.67 ± 7.75 (N=3)	5.67 ± 1.76 (N=3)	6.00 ± 1.53 (N=3)	0.00 ± 0.00 (N=3)	0.00 ± 0.00 (N=3)	0.33 ± 0.33 (N=3)	0.00 ± 0.00 (N=3)	82.67 ± 0.33 (N=3)	82.33 ± 6.33 (N=3)
Wet	Jul–Oct 2013	10.50 ± 2.66 (N=4)	13.67 ± 5.24 (N=3)	7.00 ± 3.81 (N=4)	2.33 ± 2.33 (N=3)	0.25 ± 0.25 (N=4)	0.00 ± 0.00 (N=3)	0.50 ± 0.25 (N=4)	2.33 ± 1.86 (N=3)	81.75 ± 4.77 (N=4)	81.67 ± 5.90 (N=3)

Remarks:

- Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*) and solid outline around the data.

Table 5-8 Differential leukocyte count (Mean ± S.E.M.) of female immunized *Fejervarya limnocharis* population caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Differential leukocyte count (%)									
		Neutrophil		Eosinophil		Basophil		Monocyte		Lymphocyte	
		Con.	Ref.	Con.	Ref.	Con.	Ref.	Con.	Ref.	Con.	Ref.
Cool dry	Jan 2013	24.33 ± 3.38 (N=3)	47.33 ± 2.33* (N=3)	19.33 ± 7.45 (N=3)	21.33 ± 2.03 (N=3)	0.00 ± 0.00 (N=3)	0.00 ± 0.00 (N=3)	4.33 ± 1.20 (N=3)	14.33 ± 2.03* (N=3)	52.33 ± 10.40 (N=3)	17.00 ± 2.08* (N=3)
Hot dry	April 2013	10.00 ± 2.08 (N=3)	9.00 ± 3.51 (N=3)	3.00 ± 2.52 (N=3)	3.67 ± 2.03 (N=3)	0.00 ± 0.00 (N=3)	0.00 ± 0.00 (N=3)	0.00 ± 0.00 (N=3)	0.33 ± 0.33 (N=3)	87.00 ± 1.53 (N=3)	87.00 ± 2.31 (N=3)
Wet	Jul–Oct 2013	7.40 ± 1.17 (N=5)	12.33 ± 2.67 (N=6)	5.40 ± 3.66 (N=5)	5.17 ± 1.78 (N=6)	0.00 ± 0.00 (N=5)	0.33 ± 0.33 (N=6)	1.60 ± 3.68 (N=5)	2.17 ± 0.70 (N=6)	85.60 ± 4.43 (N=5)	80.00 ± 3.79 (N=6)

Remarks:

- Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*) and solid outline around the data.

After calculation for neutrophil: lymphocyte ratio (N: L ratio), overall, N: L ratio of the frogs caught from the contaminated site tend to be relatively stable throughout the year, while significant seasonal differences were found in frogs caught from the reference site. Both group of non-immunized and immunized frogs caught from the reference site showed significant seasonal differences in N: L ratio in both male and female frogs caught from reference site (one-way ANOVA, $p<0.05$; Table 5-9 – 5-12) with highest ratio in cool dry season. During this period (cool dry season), the N: L ratio of frogs caught from the reference site were significantly higher than those of the contaminated site frogs in most group (Student t-test, $p<0.05$; Figure 5-6, 5-7 and 5-8 respectively). Although, N: L ratio of male non-immunized frog was not significantly different between site trend of higher N: L ratio in the reference site frog was still evident (Mann-Whitney rank sum test, $p>0.05$; Figure 5-5), potentially suggested suppression of immune function in the contaminated site frogs.

Table 5-9 Neutrophil: lymphocyte ratio (N: L ratio) (Mean ± S.E.M.) of male non-immunized *Fejervarya limnocharis* populations caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Neutrophil: lymphocyte ratio (N: L ratio)	
		Contaminated site	Reference site
Cool dry	Jan 2013	0.51 ± 0.14 (N=3)	1.47 ± 0.24 ^{A} (N=3)
Hot dry	April 2013	0.13 ± 0.01 (N=3)	0.12 ± 0.02 ^{B} (N=3)
Wet	Jul–Oct 2013	0.34 ± 0.13 (N=6)	0.37 ± 0.11 ^{B} (N=6)

Remarks:

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

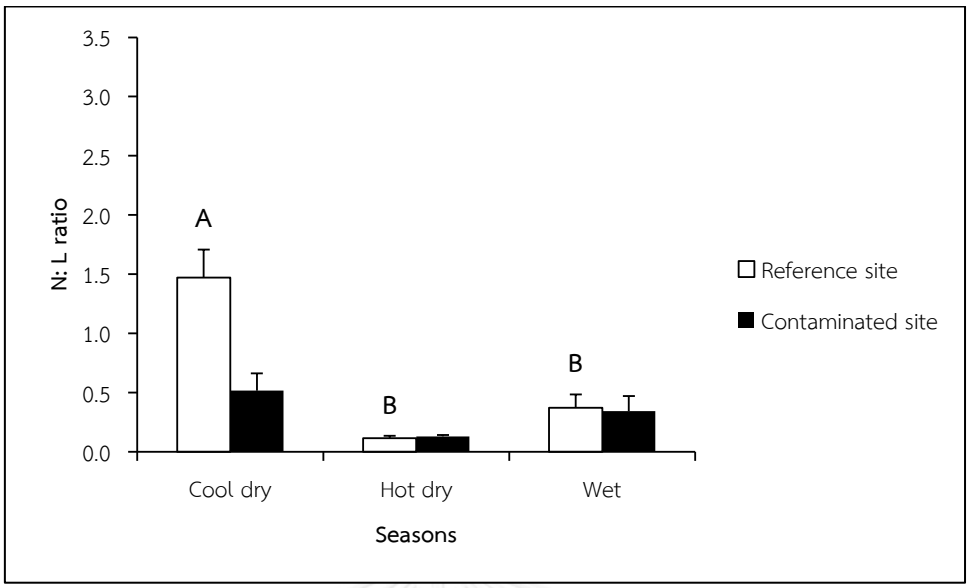


Figure 5-6 Neutrophil: lymphocyte ratio (N: L ratio) of male non-immunized frogs *Fejervarya limnocharis* from reference and contaminated sites at Nan Province, Thailand

Remarks:

- Significant seasonal-related difference (one-way ANOVA, $p<0.05$) is indicated by different letter.

Table 5-10 Neutrophil: lymphocyte ratio (N: L ratio) (Mean ± S.E.M.) of female non-immunized *Fejervarya limnocharis* populations caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Neutrophil: lymphocyte ratio (N:L ratio)	
		Contaminated site	Reference site
Cool dry	Jan 2013	0.35 ± 0.14 (N=3)	1.73 ± 0.70 $^{*, A}$ (N=3)
Hot dry	April 2013	0.17 ± 0.04 (N=3)	0.20 ± 0.12 ^{B} (N=3)
Wet	Jul–Oct 2013	0.50 ± 0.33 (N=6)	0.20 ± 0.10 ^{B} (N=4)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

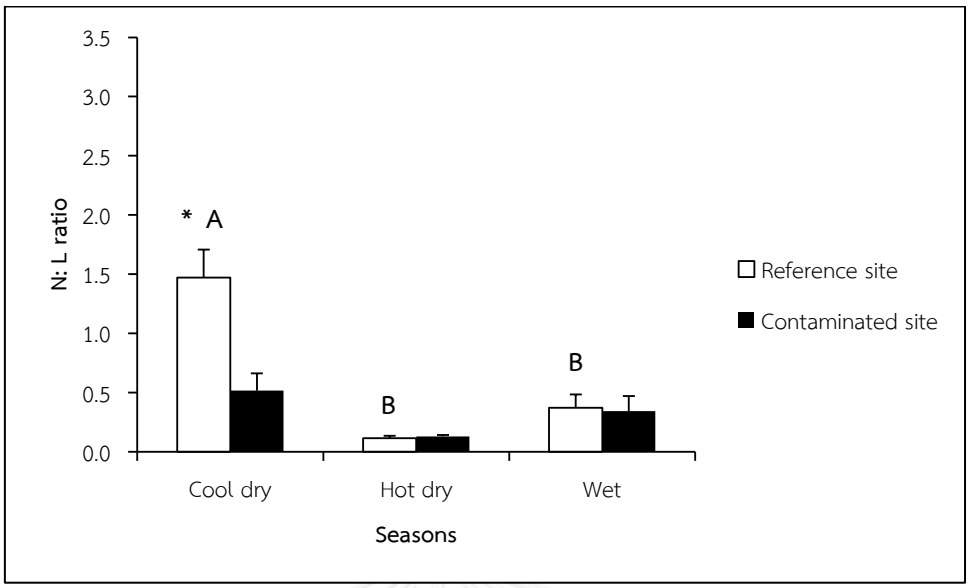


Figure 5-7 Neutrophil: lymphocyte ratio (N: L ratio) of female non-immunized *Fejervarya limnocharis* from reference and contaminated sites at Nan Province, Thailand

Remarks:

- Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*).

- Significant seasonal difference (one-way ANOVA, $p<0.05$) is indicated by different letter.

Table 5-11 Neutrophil: lymphocyte ratio (N: L ratio) (Mean ± S.E.M.) of male immunized *Fejervarya limnocharis* populations caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Neutrophil: lymphocyte ratio (N:L ratio)	
		Contaminated site	Reference site
Cool dry	Jan 2013	0.75 ± 0.35 (N=3)	2.21 ± 0.26 $^{*, A}$ (N=3)
Hot dry	April 2013	0.14 ± 0.03 (N=3)	0.16 ± 0.11 ^{B} (N=3)
Wet	Jul–Oct 2013	0.13 ± 0.04 (N=4)	0.18 ± 0.08 ^{B} (N=3)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

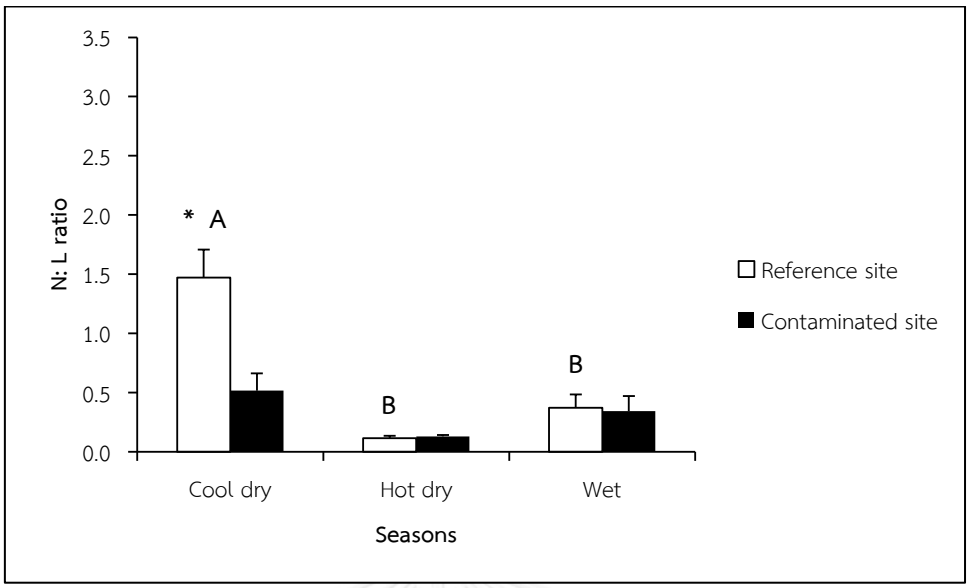


Figure 5-8 Neutrophil: lymphocyte ratio (N: L ratio) of male immunized frogs *Fejervarya limnocharis* from reference and contaminated sites at Nan Province, Thailand

Remarks:

- Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*).

- Significant seasonal difference (one-way ANOVA, $p<0.05$) is indicated by different letter.

Table 5-12 Neutrophil: lymphocyte ratio (N: L ratio) (Mean ± S.E.M.) of female immunized *Fejervarya limnocharis* population caught from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/year	Neutrophil: lymphocyte ratio (N:L ratio)	
		Contaminated site	Reference site
Cool dry	Jan 2013	0.17 ± 0.03 (N=3)	2.88 ± 0.41 $^{*, A}$ (N=3)
Hot dry	April 2013	0.12 ± 0.02 (N=3)	0.11 ± 0.04 ^{B} (N=3)
Wet	Jul–Oct 2013	0.09 ± 0.02 (N=6)	0.17 ± 0.05 ^{B} (N=6)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student t-test, $p<0.05$).

- Difference in superscript letter indicates significant difference between sampling periods within the same site (one-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

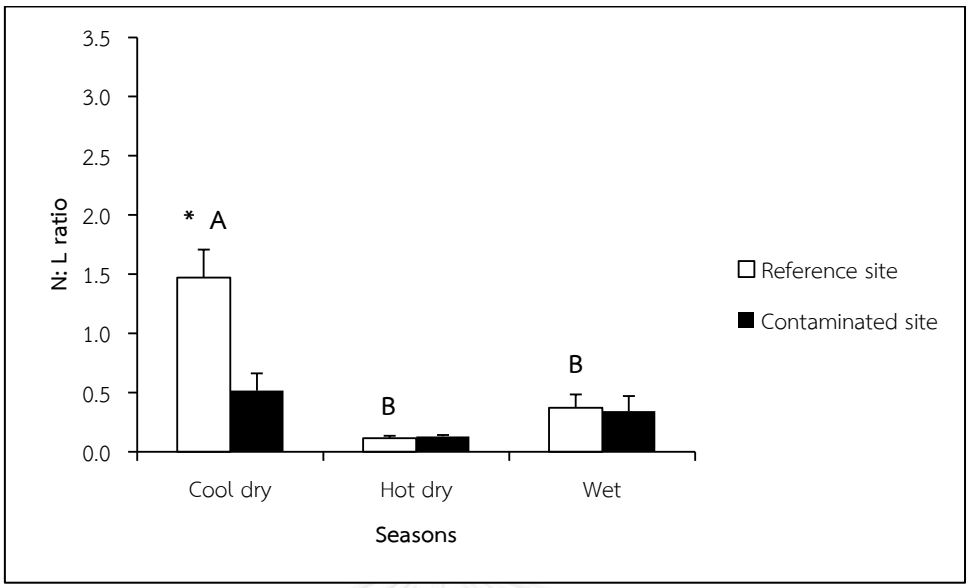


Figure 5-9 Neutrophil: lymphocyte ratio (N: L ratio) of female immunized *Fejervarya limnocharis* from reference and contaminated sites at Nan Province, Thailand.

Remarks:

- Significant site-related difference (t-test, $p<0.05$) is indicated by an asterisk (*).

- Significant seasonal difference (one-way ANOVA, $p<0.05$) is indicated by different alphabet.

3. Correlation between Corticosterone Level versus Neutrophil: Lymphocyte Ratio (N: L Ratio)

Correlation analysis of corticosterone level versus N: L ratio showed that there was no significant correlation between corticosterone level versus N: L ratio (Spearman Rank Order Correlation, $r=-0.145$, $p>0.05$).

Discussions

Recent evidence suggested that xenobiotic contaminants in environment can induce increases in corticosterone levels (Gendron et al., 1997; Hopkins et al., 1999). In this study, site-related difference in plasma corticosterone level was not found in the natural population of frogs (non-immunized frogs). However, after immunization, the enhanced immunologic state leaded to significant site-related difference in these frogs with a distinct pattern between male and female frogs. At this stage, it is not yet conclusive whether the different corticosterone level was due to internal factor (i.e. immunologic function) or external factor (i.e. environmental contamination).

It is importance to note that the immunized frogs were kept in plastic aquaria for 9 days during immunization. Since baseline corticosterone levels correlate with periods of activity and levels of corticosterone could increase immediately before or during the active period. (Pancak and Taylor, 1983; Thurmond et al., 1986; Chiba and Aoki, 1987), keeping frogs in captivity could disturb the corticosterone profile and obscure the observation, to some extent. In addition, since rhythmicity of corticosterone secretion in this frog species is unknown, taking blood sample at different time at the day could yield high variation in corticosterone level. In this study frog dissection was performed from dawn until dusk to cope with high number of sample. Although the attempts to dissect one sample from every groups at a

given time were strictly executed, there were still chances of high individual variation with in each group due to rhythmicity of corticosterone level.

Although evidence of non-specific immune response in term of corticosterone level was not conclusive, neutrophil: lymphocyte ratio showed a unique sign of different non-specific immune response between site. Interestingly, for both non-immunized and immunized frogs, N: L ratio of the reference site frogs was markedly elevated than those of the contaminated site frogs in cool dry season. In this period, agricultural activity was minimal at the reference site, while agricultural activity (including the use of agrochemicals) was present in contaminated site. As a result, lower N: L ratio in the contaminated site may suggest that agricultural activity presented in contaminated site in cool dry season could suppress an immune response of frog inhabited in this area.

Since there were limited member of reports on amphibian leukocyte profiles and because the profiles seem to change in response to a variety of environmental and biological factors (Duellman and Treub, 1986). For example, there was an increase in the average percentage of neutrophils and a decrease in the mean percentage of eosinophils of the leopard frog *Rana pipiens* kept in the cold temperature (Maniero and Carey, 1997). Davis et al. (2010) also reported that *Batrachochytrium dendrobatidis* (Bd) infections elicit increases in glucocorticoid hormones, which can cause increased numbers of circulating neutrophils and lower numbers of eosinophils of Bullfrogs (*Rana catesbeiana*). It is thus too early to make a generalization about how leukocyte profiles were affected by herbicide utilization. However, with the easiness of technique and potential implication in

Immunotoxicology, validation of leukocyte profiles as alternative biomarkers of immune function is recommended.

Correlation between stress hormone level and N: L ratio was not found in this study. Since pattern of corticosterone secretion in this species is not known to be nocturnal, diurnal or constant, it is possible that blood sampling at different time of day as performed in this study could result in high individual variation in corticosterone level, overcoming the subtle site-related difference in these frogs. Therefore, taking blood sample of every individual at the same period during the day is recommended.

Conclusion

This study examined the stress (corticosterone) hormone and leukocyte (N: L ratio) responses to environmental stress in term of herbicide utilization. There was no site-relate difference in corticosterone level between reference and contaminated site frog. However, the significant difference in corticosterone level was observed in frogs after immunization, possibly due to change in physiological conditions during captivity. Importantly, N: L ratio or an indicator of non-specific immune response of reference site frog was significantly higher than frog caught from contaminated site. This might indicate that herbicide utilization in agricultural area could suppress non-specific immune response of the frog.

CHAPTER 6
HEPATIC MELANOMACROPHAGE OF RICE FROG *Fejervarya limnocharis* LIVING IN AGRICULTURAL AREAS AT NAN PROVINCE

Introduction

Environmental contamination may cause adverse responses in animals at different structural levels, including cells, tissues, and organs. These effects depend on a variety of factors, such as the type of contaminant and its concentration, the rate of exposure, and the susceptibility of the organisms (Fenoglioa et al., 2005). Certain xenobiotics may have direct or indirect adverse effects by generating bioactive molecules intracellularly (including free radicals), causing functional and structural alterations at both nuclear and cytoplasmic levels (Williams and Iatropoulos, 2002). Hepatocytes and kupffer cells (KCs) in liver of vertebrates plays a fundamental role in biotransformation processes of xenobiotics which involve in both through enzymatic and non-enzymatic mechanisms. Therefore, the liver of ectothermic vertebrates, such as amphibian, is the most suitable organ for evaluating response against environmental pollutants.

Kupffer cells in the liver of ectotherms contain melanic pigment in cytoplasm, making them known as melanomacrophages (Fenoglioa et al., 2005). Melanomacrophage centers (MMCs) or macrophage aggregates (MAs) or macro-melanophage center (Grier and Taylor, 1998; Zorita et al., 2008) are accumulations of pigment-bearing macrophages which are a prominent feature observed in spleen, kidney, and liver of fishes (Agius, 1980) and in liver of frogs (Loumbourdis and Vogiatzis, 2002; Boncompagni et al., 2004). Pigments collected by

melanomacrophages, including hemosiderin, lipofuscin and/ or melanin could reflect possible pathological processes and tissue destruction. Hemosiderin, an iron-containing pigment, is a breakdown product of red blood cells. Lipofuscin or ceroid is formed from the oxidative polymerization of polyunsaturated fatty acids and protein (Agius, 1985; Wolke et al., 1985). Their function has been associated with 1) inflammatory and humoral responses; 2) cell and compound storage, destruction, and detoxification of exogenous and endogenous waste products; and 3) iron recycling (Montero et al., 1999; Fournie et al., 2001). Melanomacrophage centers in fish are believed to be primitive analogues of germinal centers in aves and mammals (Ellis, 1980; Agius and Agbede, 1984; Martinez et al., 1994). MMCs frequency and pigment composition in fishes are dependent on species, health status of the fish (Agius, 1979; Herraez and Zapata, 1986; Herraez and Zapata, 1987), nutrition (Micale and Perdichizzi, 1990; Montero et al., 1999), and age (Agius, 1981; Brown and George, 1985; Blazer et al., 1987).

In a number of studies in fishes, MMCs were used as a possible index of environmental stress (Blazer et al., 1987; Couillard and Hodson, 1996; Meinelt et al., 1997; Fournie et al., 2001; Facey et al., 2005) and later on were recognized as potentially useful biomarkers in ecotoxicology (Wolke et al., 1981; Wolke et al., 1985). Numerous studies in fishes documented an increase in their number, size or hemosiderin content in fish collected at contaminated sites compared to those collected at reference sites. Although MMC can be observed histologically, it has been suggested that MMCs may be immunotoxicologic biomarkers (Blazer et al., 1987; Weeks et al., 1992).

In amphibian, the pigmented cell of the liver is a very plastic element whose melanosome content and distribution undergo alterations with oxygen supply (Frangioni et al., 2000), seasonal changes (Barni et al., 1999; Barni et al., 2002), and xenobiotic exposure (Loumbourdis and Vogiatzis, 2002). Since MMCs in amphibian liver may respond to xenobiotics in a similar manner to fish MMCs. Livers of marine toads (*Bufo marinus*) collected in sites contaminated with pesticides and heavy metals exhibited an increased number of melanomacrophages compared to tissues of toads collected at control sites (Linzey et al., 2003). Reduced abundance of hepatic melanomacrophage aggregates in northern leopard frogs exposed to atrazine was evident and correlated with increased infections (R^2=0.519, $p<0.05$) by larval trematodes. This suggested that MMCs might form part of an important immune response to the trematode infections, and that atrazine may suppress this response (Rohr et al., 2008c).

In this study, the rice frog, *Fejervarya limnocharis*, was used as a sentinel species of environmental stress from the herbicide contamination. Stable population of *F. limnocharis* can be found in the agriculture areas where intensive and continuous utilization of herbicide were evidenced, making it susceptible to long term exposure and accumulation of xenobiotics (Othman et al., 2009). Prior observation indicated that utilization of herbicides in agricultural area could lead to contamination and adverse health effects in the rice frog *F. limnocharis* living in the area. Since herbicide contamination may influence disease emergence by acting upon an immune system of amphibian, it is interesting to investigate potential effects of herbicide contamination on non-specific immune responses of the rice frog living in agricultural areas.

Objective

Using the rice frogs, *F. limnocharis*, living in agricultural areas with different degree of herbicide utilization at Nan Province as a model, this study aimed to examine the use of melanomacrophage and melanomacrophage center as hepatic biomarkers for non-specific immune response.

Materials and Methods

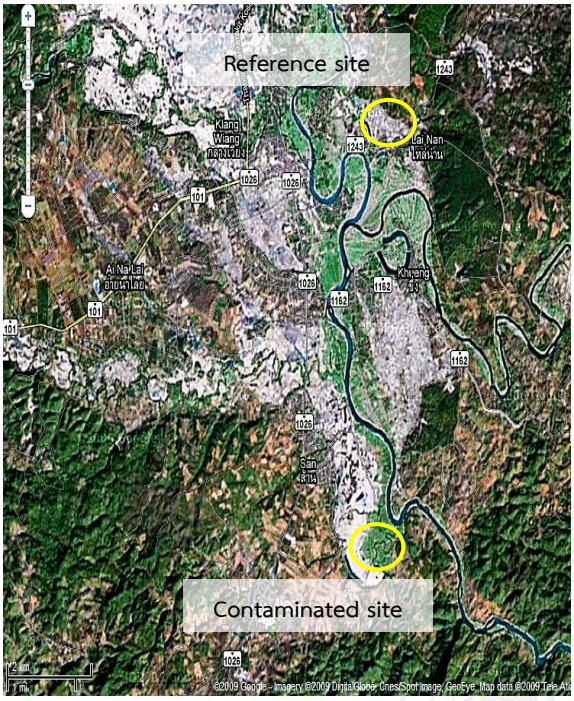
1. Study Site

The study sites located in Wiang Sa District, Nan Province included

1) a contaminated site which is an agricultural area with intensive herbicide usage located in San Subdistrict (47Q 0687729 UTM 2054283)
2) a reference site which is an organic agricultural area with no history of herbicide usage for almost 10 years located in Lai-nan Subdistrict (47Q 0686779 UTM 2047187) (Figure 6-1).

Prior study indicated that atrazine residue (0.15 mg/L) was found in surface water at the contaminated site during late dry season while no herbicide residue was found in the reference site in any season (Maneein et al., 2011; Thammachoti et al., 2012). Further analyses showed that frog from the contaminated site tended to have higher level of glyphosate and markedly higher level of atrazine and paraquat in the tissue compared to those in the reference site (Thammachoti et al., 2012).

Since data on herbicide residues in frog samples at Nan Province was readily available (Thammachoti, 2012), further analysis for correlation between herbicide residue and change in hepatic biomarkers could be performed using frog samples caught from the study sites in previous study by Thammachoti (2012)



UTM 0687729 2054283 (zone line 47Q)

UTM 0686779 2047187 (zone line 47Q)

Figure 6-1 Study sites included a reference site which is a paddy field in Lai-nan Subdistrict with no history of herbicide usage and a contaminated site in San Subdistrict with intensive herbicide usage.
(Thammachoti, 2012)

2. Rice Frog Collection

The rice frogs were field-collected by Thammachoti (2012) in July 2010 (late wet period), October 2010 (early dry period), January 2011 (late dry period) and April 2011 (early wet period) from two study sites. Wet and dry seasons in this study were determined based on the climate diagram (Figure 6-2) plot between mean temperature and total rainfall (Walter et al., 1975). For each period, 20 adult frogs (10 males and 10 females) were collected by hand at night during visual encounter surveys (Crump and Scott, 1994). After transportation to the laboratory at Chulalongkorn University Forest and Research Station, Nan Province, frogs were euthanized by immersion into 0.5% Ethyl 3-aminobenzoate methanesulfonate salt solution (MS-222; Sigma-Aldrich, St.Louis, MO, USA). Ten livers of the remaining frogs (5 male and 5 female frogs) were dissected and fixed in 10% neutral buffer formalin for histological analysis.

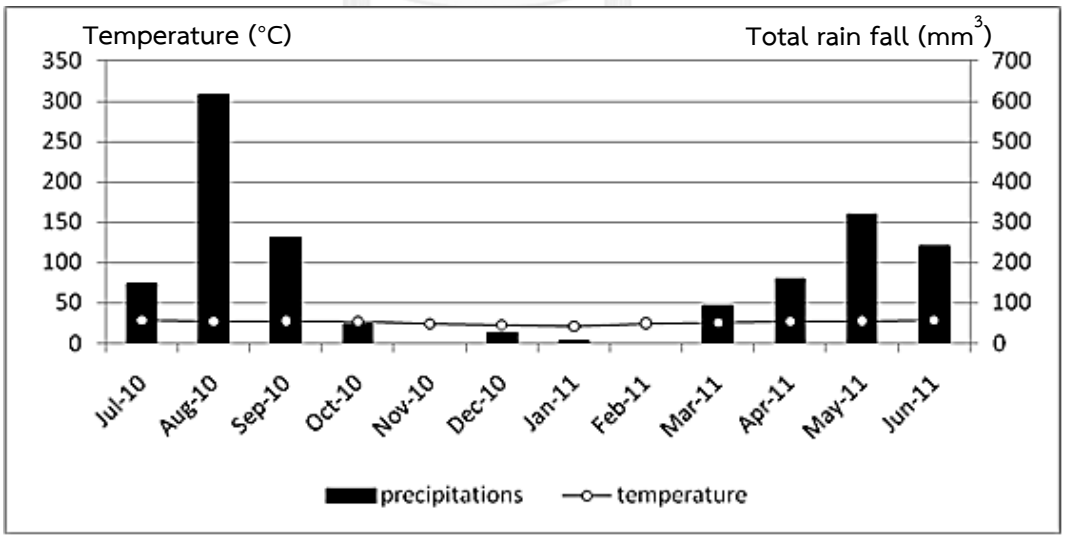


Figure 6-2 Climograph of paddy fields in Nan Province during sampling period (July 2010 – June 2011)
(Thammachoti, 2012)

3. Melanomacrophage and Melanomacrophage Center (MMC) in Liver of the Rice Frogs

Fixed liver obtained from Thammachoti (2012) study of male (n=5) and female (n=5) rice frogs collected in late wet period (July 2010), early dry period (October 2010), late dry period (January 2011) and Early wet period (April 2011) from both reference and contaminated sites (Thammachoti, 2012) were randomly selected to use for examination on melanomacrophage and MMC. Liver tissues were processed through paraffin method (Humason, 1979), cut at 5 µm with a rotary microtome (Leica RM2165)) and stained with Delafield's hematoxylin and eosin (H&E; Humason (1979)). Numbers of melanomacrophage and MMC in liver were counted using ocular grid (Olympus eyepiece micrometer) under 100X magnification of a light microscope (Olympus CH-2).

4. Statistical analyses

Data were tested for normal distribution and homogeneity of variance. Mean comparison between sexes and sites was determined using Student's t-test. Difference among seasons was analyzed by one-way ANOVA followed by Student-Newman-Keuls Method. Correlation of melanomacrophage and MMC numbers vs. herbicide residues (Thammachoti, 2012) was determined using Pearson's correlation.

Results

1. Melanomacrophage and melanomacrophage center (MMC) in liver of the rice frogs

Melanomacrophage and MMCs are focal accumulations of pigmented macrophages in liver, spleen and kidney of fish (Agius and Roberts, 2003) and in liver of frog (Loumbourdis and Vogiatzis, 2002). These pigment cells belong to the exocutaneous pigmentary system of the lower vertebrates (Breathnach, 1988). In this study, it was found that melanomacrophages and MMCs were found in liver of the rice frog from both reference and contaminated sites (Figure 6-3 – 6-6).

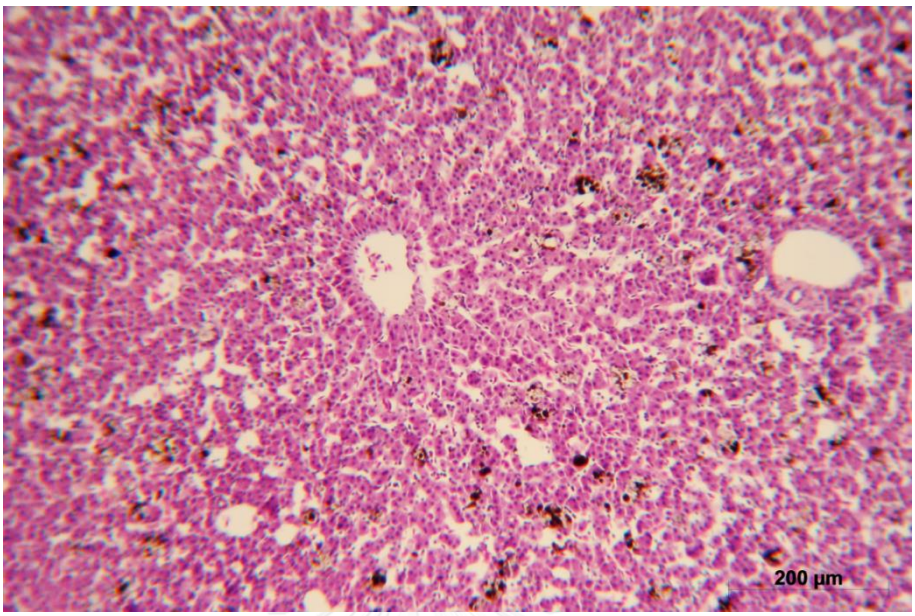


Figure 6-3 Micrographs of liver (H&E staining) of the rice frog, *Fejervarya limnocharis*, caught from the reference site (100X) in Nan Province, Thailand

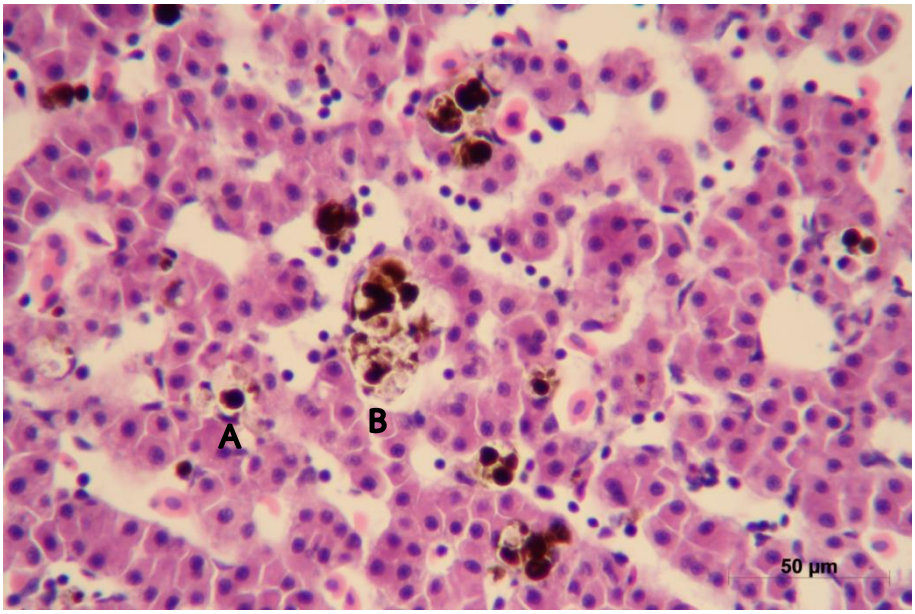


Figure 6-4 Micrographs of liver (H&E staining) of the rice frog, *Fejervarya limnocharis*, caught from the reference site (400X) in Nan Province, Thailand
Melanomacrophages (A) and melanomacrophage centers (MMCs; B) are shown.

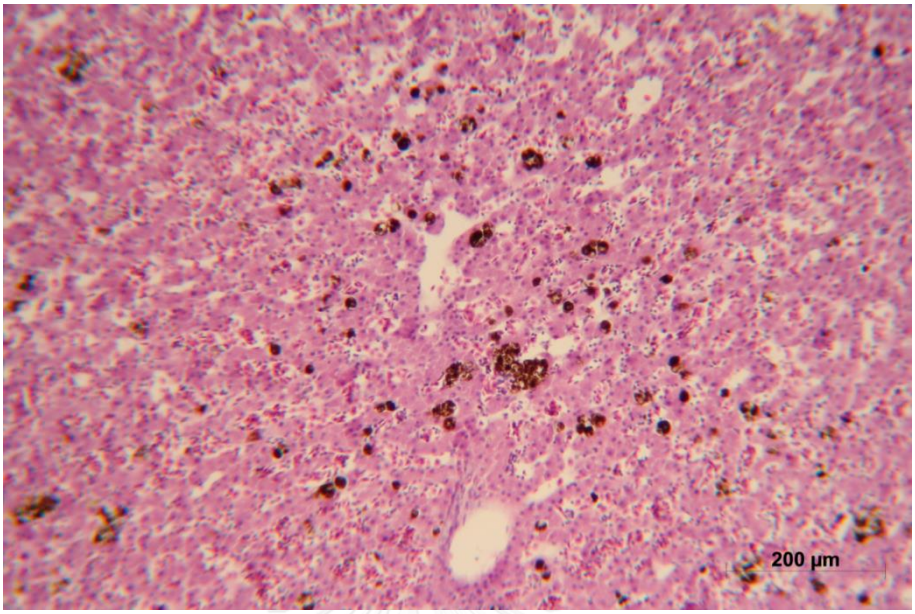


Figure 6-5 Micrographs of liver (H&E staining) of the rice frog, *Fejervarya limnocharis*, caught from the contaminated site (100X) in Nan Province, Thailand

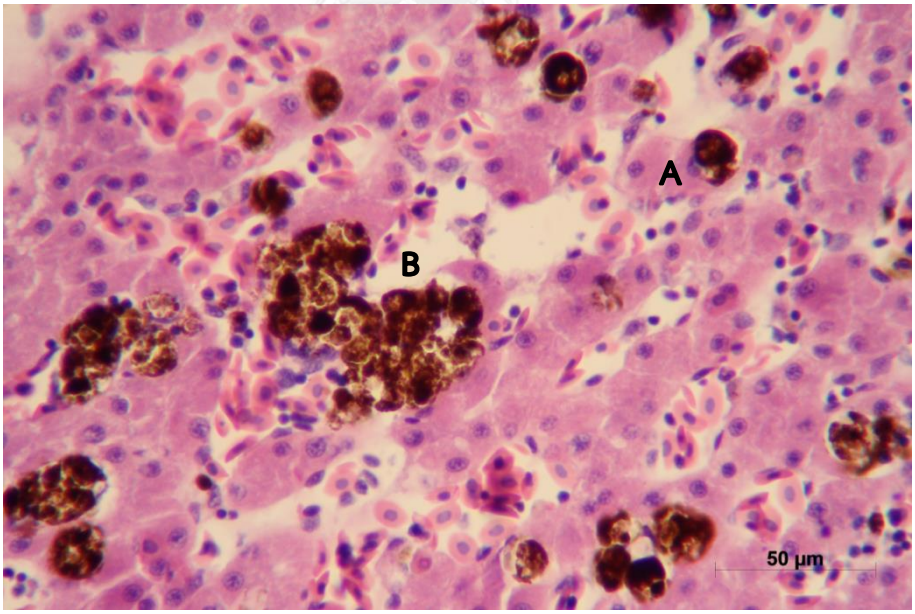


Figure 6-6 Micrographs of liver (H&E staining) of the rice frog, *Fejervarya limnocharis*, caught from the contaminated site (400X) in Nan Province, Thailand. Melanomacrophages (A) and melanomacrophage centers (MMCs; B) are shown.

To determine if sex had a significant effect on this parameter, comparison for number of melanomacrophages and MMCs was made. No significant sex-related difference in these numbers was found (Student's t-test, $p>0.05$, data not shown). However, there were significant site-related differences in these numbers, with the higher numbers of melanomacrophages and MMCs in the contaminated site frog (Student t-test, $p<0.05$), except in late dry period (January 2011) (Figure 6-7). Furthermore, there were significant seasonal differences in these numbers in both reference and contaminated site frogs (Table 6-1), which may relate to the difference in agricultural activities of both study sites (data not shown).

Table 6-1 Numbers (Mean ± S.E.M.) of melanomacrophages and melanomacrophage centers (MMCs) in liver of the rice frog, *Fejervarya limnocharis*, caught from the reference site and the contaminated site during 2010-2011 in Nan Province, Thailand

Period	Month/Year	Reference site (MMCs/ mm^2)	Contaminated site (MMCs/ mm^2)
Late wet	July 2010	78.27 ± 16.07 ^{A} (N=10)	141.26 ± 17.26 $^{*, a}$ (N=10)
Early dry	October 2010	15.82 ± 3.03 ^{C} (N=10)	62.25 ± 7.77 $^{*, c}$ (N=10)
Late dry	January 2011	62.23 ± 6.31 ^{AB} (N=10)	55.27 ± 4.32 ^{c} (N=10)
Early wet	April 2011	41.78 ± 6.78 ^{BC} (N=10)	106.97 ± 7.80 $^{*, b}$ (N=10)

Remarks:

- Significant site-related difference within the same period (Student t-test, $p<0.05$) is indicated by an asterisk (*).

- Significant seasonal difference within the same site (one-way ANOVA, $p<0.05$) is indicated by different superscript letters. (Significant seasonal-related difference within contaminated site is indicated by the small letter and significant seasonal-related difference within reference site is indicated by the capital letter).

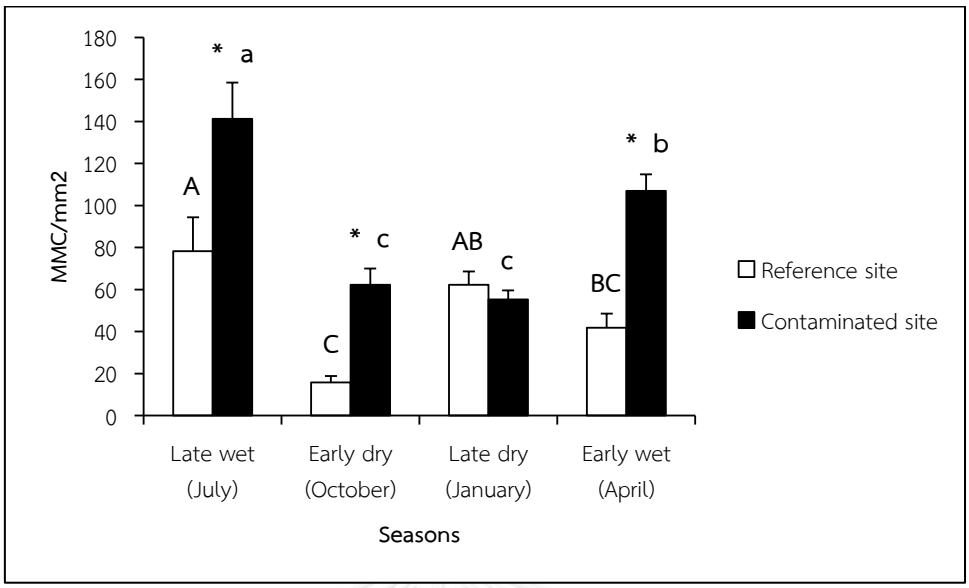


Figure 6-7 Numbers of melanomacrophages and melanomacrophage centers (MMCs) in liver of the rice frog, *Fejervarya limnocharis*, caught from the reference site and the contaminated site during 2010-2011 in Nan Province, Thailand

Remarks:

- Significant seasonal difference within the same site (one-way ANOVA, $p<0.05$) is indicated by different superscript letters. (Significant seasonal-related difference within contaminated site is indicated by the small letter and significant seasonal-related difference within reference site is indicated by the capital letter).

- Significant site-related difference within the same period (Student t-test, $p<0.05$) is indicated by an asterisk (*).

2. Correlation of melanomacrophage and MMC numbers vs. herbicide residues

Correlation analysis of melanomacrophage and MMCs numbers versus herbicide residues in frog tissue (Table 6-2; (Thammachoti, 2012)) showed significant correlation between numbers of melanomacrophages and MMCs vs. atrazine in the rice frog (Pearson's correlation coefficient = 0.586, $p<0.05$; Table 6-3). However, there was no significant correlation between numbers of melanomacrophages and MMCs vs. glyphosate (Pearson's correlation coefficient = -0.479, $p>0.05$) and paraquat (Pearson's correlation coefficient = -0.119, $p>0.05$) residues.

Table 6-2 Herbicide residues (Mean ± S.E.M.) in tissues of the rice frog *Fejervarya limnocharis* caught from the reference site and the contaminated site during 2010-2011 in Nan Province, Thailand (Thammachoti, 2012)

Herbicides	Period	Month/Year	Reference site (ng/g)	Contaminated site (ng/g)
Atrazine	Late wet	July 2010	4.22 ± 1.72 (N=6)	14.10 ± 5.83 (N=6)
	Early dry	October 2010	5.11 ± 2.61 (N=6)	4.12 ± 1.61 (N=6)
	Late dry	January 2011	7.26 ± 1.96 (N=6)	9.06 ± 2.32 (N=6)
	Early wet	April 2011	3.62 ± 0.50 (N=6)	7.51 ± 3.73 (N=6)
Glyphosate	Late wet	July 2010	4.91 ± 1.17 (N=6)	5.48 ± 2.74 (N=6)
	Early dry	October 2010	8.26 ± 2.64 (N=5)	10.90 ± 6.29 (N=5)
	Late dry	January 2011	6.48 ± 0.77 (N=6)	9.07 ± 4.53 (N=6)
	Early wet	April 2011	5.29 ± 0.55 (N=5)	6.40 ± 3.69 (N=5)
Paraquat	Late wet	July 2010	59.78 ± 6.30 (N=6)	64.49 ± 10.39 (N=6)
	Early dry	October 2010	70.07 ± 15.30 (N=5)	113.51 ± 27.81 (N=5)
	Late dry	January 2011	49.73 ± 4.67 (N=6)	115.18 ± 40.20 (N=5)
	Early wet	April 2011	47.88 ± 7.74 (N=5)	66.92 ± 20.39 (N=5)

Table 6-3 Pearson's correlation between herbicide residues in tissues and number of melanomacrophage and melanomacrophage center (MMC) in liver of rice frog, *Fejervarya limnocharis*, living in agricultural area at Nan Province, Thailand

Data	Atrazine	Glyphosate	Paraquat	Number of MMCs
Number of MMCs	0.586 p=0.017 *	-0.400 p=0.124	-0.050 p=0.853	1.000 p=0.000
Paraquat	0.0489 p=0.857	0.738 p=0.001 *	1.000 p=0.000	
Glyphosate	-0.144 p=0.594	1.000 p=0.000		
Atrazine	1.000 p=0.000			

Remarks:

- Significant between parameters (n=16) is indicated by an asterisk (*)

Discussions

Prior study showed that only atrazine residue was present in the detectable amount in water sample of the agricultural area at Nan Province while levels of glyphosate and paraquat were below the limit of detection (Maneein et al., 2011; Thammachoti, 2012). Atrazine contamination was found in late dry season (January 2011), indicating that pattern of herbicide utilization could be a major contributing factor to contamination in addition to pattern of weather or season. Further study by Thammachoti (2012) indicated that these herbicides (atrazine glyphosate and paraquat) were contaminated in frog tissues at both reference and contaminated sites. Albeits its similar presence in frog tissue, the level of atrazine and paraquat contamination in the contaminated site frogs were still significantly higher than those from the reference site. This confirms the assumption that an intensive use of herbicides could lead to contaminate in tissue of the frog living in the paddy fields (Thammachoti, 2012). Although atrazine contamination is relatively low, it is of importance to note that atrazine is a known endocrine disrupting chemicals that may exert its effect, including alters reproductive system of amphibians, at relatively low level of contamination (Hayes et al., 2002).

In this study, influence of herbicide utilization on immune system of the rice frogs was examined. The results showed that there were significant seasonal and site-related differences in numbers of melanomacrophage and MMC in liver of the rice frogs. This suggests that seasonal and site-related difference in herbicide utilization tend to affect frog's immune system in agricultural areas. For seasonal difference, it was well known that liver of the frog is a very plastic organ in which both the epithelial and the histiocytic components are very sensitive to certain

annual biological rhythms (i.e. reproduction). Liver melanomacrophages of *Rana esculenta* tended to show difference in metabolically and cytokinetically active cell population during the annual cycle. This phenomenon is probably regulated by highly integrated mechanisms responsible for maintaining a functional homeostatic balance during the different adaptation responses (Barni et al., 2002). In addition, MMC's function has been linked with cell and compound storage, destruction, and detoxification and iron recycling (Agius and Roberts, 2003). MMCs of teleost fish were reported to trap and retain antigens during the immune response and were closely associated with immunoglobulin-secreting cells (Vigliano et al., 2006). Since MMCs could play roles in immunity (inflammatory and humoral responses; Agius and Roberts (2003)), an increase in number and area of MMCs could be sensitive to environmental stressors.

Unfortunately, there were only a few studies in regard to the use of numbers of MMCs as immunological biomarkers in the frogs and other amphibians (Barni et al., 2002; Loumbourdis and Vogiatzis, 2002; Grassi et al., 2007) when compared to fishes. Nevertheless, pigmented macrophage accumulations were used as potential marker of fish health (Blazer and Dethloff, 2000). Studies about MMCs as biomarkers in many fish species demonstrated that the occurrence of MMCs may vary depending on many factors namely the nutritional status, health or size of a particular fish species (Agius, 1979; Agius, 1980; Agius and Roberts, 1981; Wolke et al., 1985). The poor health fish or fish with nutritional deficiencies and larger fish tend to have more or larger MMCs. Moreover, the number and/or size of MMCs in many fish increase with age (Brown and George, 1985; Blazer et al., 1987). Importantly, numerous studies had documented an increase in their number, size or hemosiderin content in

fish collected at contaminated sites compared to those collected at reference sites. MMCs were used as a potentially sensitive biomarkers of contaminant exposure and a potential immunotoxic biomarkers (Blazer et al., 1997; Matavulj et al., 2005). Since there were only a few extensive, controlled attempts to produce MMCs or to study their kinetics by chronic exposure to contaminants known to exist in polluted environments, the use of MMCs as biomarkers of vertebrate health and environmental degradation should be further examined using the systematic controlled experiment (Blazer and Dethloff, 2000).

Conclusion

Melanomacrophage and melanomacrophage center (MMCs) have been recommended as potentially sensitive biomarkers of contaminant exposure because their functions included immune response and detoxification. This current finding of significant seasonal and site-related differences in numbers of melanomacrophages and MMCs and correlation between numbers of melanomacrophages and MMCs versus atrazine residue suggested potential effect of herbicide on non-specific immune response of the frog in herbicide utilized agricultural area.

CHAPTER 7
GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

Potential influence of herbicide utilization on immune response of the rice frog living in agricultural areas was examined in this study. Agricultural area in Nan Province in the northern part of Thailand was chosen as a study area since most of agrochemicals used in agricultural activities of this area was herbicides with atrazine, glyphosate and paraquat being the most commonly used chemicals (Thammachoti, 2012).

A reference and a herbicide contaminated agricultural areas were located in Wiang Sa District, Nan Province. The reference site (at Lai-Nan Subdistrict) located about 7.2 km north of the contaminated site is an organic agricultural area with no history of herbicide utilization for more than 10 years. Whereas, the contaminated site (San Subdistrict) is an agricultural area with an intensive and prolonged herbicide utilization. In addition to difference in herbicide utilization, other factors including landscape, climate and other physical factors of these two sites were similar, and their effects of the populated rice frogs should be minimal.

To determine the potential influence of herbicides on immune response and morphology of rice frog, sample of the rice frog was collected on every 3 months covering cool dry, hot dry and wet seasons from these two study sites and subjected to examination using multiple parameters. Results of this study can be divided into three parts corresponding to morphology, specific and non-specific immune response. The summay of the results are shown in Table 7.1

In the first part of this study, frogs were examined for condition factor as well as gravimetric and morphometric parameters and compared between two study sites. It was found that the condition factor, an indicative of overall health, was reduced in the agricultural area where herbicide was intensively used. In addition, significant difference in splenosomatic index indicated that frog from these study sites might exhibit different in immunological status. The markedly difference in female gonadosomatic index indicated that the contaminated site frog has enhanced ovarian growth even in the non-breeding season (cool dry period), while higher GSI in male frog from the contaminated site might indicated pathological alteration of the testis. These results suggested that the use of herbicides in agricultural area could influence on health and morphology of internal organs of the rice frog living in this area.

To examine the influence of herbicides on specific immune response of the rice frog, the second part of this study was carried out. The delayed-type hypersensitivity (DTH) responses showed that frogs caught from the reference site have significantly higher responses than frogs from the contaminated site. In other word, the results indicated immunosuppression in frogs from the contaminated site. Although, there are species different in the PHA skin-swelling, this result suggested that the PHA method could provide a robust measure of anuran immune response. The DTH responses could be used as of a useful tool for monitoring immune response of the rice frog in order to evaluate the environmental stress.

The third part of this study was carried out to determine the influence of herbicides on non-specific immune response of the rice frog. This study examined the stress (corticosterone) hormone and leukocyte (N: L ratio) responses to

environmental stress in term of herbicide utilization. There was no site-relate difference in corticosterone level between reference and contaminated site frog. N: L ratio, an indicator of non-specific immune response, was significantly higher in the reference site frog compared to those of frog caught from contaminated site. Although no correlation between stress hormone level and leukocyte parameter was found, the overall result suggested that herbicide utilization in agricultural activity could suppress non-specific immune response of the frog.

The final part of this study was carried out to determine the influence of herbicides on number of melanomacrophage and melanomacrophage center (MMC) in liver of the rice frog since melanomacrophage and MMC have been recommended as potentially sensitive hepatic biomarkers of contaminant exposure and non-specific immune response. The study found that there were significant seasonal and site-related differences in numbers of melanomacrophages and MMCs. Furthermore, correlation between numbers of melanomacrophages and MMCs versus atrazine residue was found, suggesting potential effect of herbicide on non-specific immune response of the frog living in herbicide utilized agricultural area. The use of numbers of melanomacrophages and MMCs to assess the environmental stress (such as herbicide) is also suggested as a useful tool for monitoring immune response of the rice frog.

Overall results suggest that the mixed and long term use of herbicides in agricultural area could influence on immune system and morphology of the rice frog inhabiting in this area. Further study on immune response of the rice frog, especially histopathology and validation of an immune function of melanomacrophage and melanomacrophage center (MMC) in liver and spleen tissue, should be performed in

order to provide a powerfully link between herbicide contamination and immune response of the surrogate vertebrates. These results could be used as a cautionary of environmental health hazards in term of immune response for other vertebrates living near the herbicide utilization area, including human.

Recommendation

1. In addition to its prior use as a sentinel for cadmium and herbicide contamination, rice frog living in agricultural areas also showed measurable immune responses to herbicide utilization. Therefore using the rice frog as sentinel species for immunotoxic effects of xenobiotic contamination is recommended.

2. Potential influence of herbicide utilization in agriculture on immune system and morphology of the rice frog provides further evidence of adverse effects of herbicide on the non-target organism. Dissemination of these findings to farmer communities is recommended in order to develop a guideline for safe agricultural practice.

Table 7-1 Summary of the site-related difference in parameters used for monitoring influence of herbicide on morphology and immune function of the rice frog *Fejervarya limnocharis* in paddy fields of Nan Province

Parameters	Results		Remarks
	Contaminated site	Reference site	
Morphometric & gravimetric analysis			
Condition factor		*	
Splenosomatic index (male)	*		
Splenosomatic index (female)	*		
Hepatosomatic index			
Renosomatic index		*	
Gonadosomatic index (testis)	*		
Gonadosomatic index (ovary)	*		
Specific immune response			
Delayed-type hypersensitivity (DTH)		*	
Non-specific immune response			
Corticosterone level (male)	*		Immunized frogs
Corticosterone level (female)		*	Immunized frogs
Neutrophil: lymphocyte ratio (N: L ratio)		*	
Hepatic melanomacrophage	*		

Remark:

- Significantly higher level (Student t-test or Mann-Whitney rank sum test, $p<0.05$) is indicated by an asterisk (*).

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APPENDIX A

Table A-1 Physical factors including relative humidity (%), temperature (°c) and soil pH from reference and contaminated agricultural areas at Nan Province, Thailand

Seasons	Month/ year	Physical factors					
		Relative humidity (%)		Temperature (°c)		Soil pH	
		Contaminated site	Reference site	Contaminated site	Reference site	Contaminated site	Reference site
Cool dry	Jan 2013	70.0	76.5	25.5	24.5	6.7	7.0
Hot dry	Apr 2013	84.5	85.5	24.0	24.5	6.8	6.7
Wet	Jul 2013	85.5	85.0	26.0	27.5	6.4	5.8
	Oct 2013	91.5	91.0	21.0	20.0	6.4	6.2

APPENDIX B

Validation of immune function of melanomacrophage and melanomacrophage center (MMC)

Immunohistochemical staining for CNA-42 was employed for an immune organ of the rice frog to determine whether melanomacrophage and MMC have an immune function similar to human follicular dendritic cells (FDCs) or antigen-presenting cell in mammals (Vigliano et al., 2006).

Using frog caught during January-October 2013, spleen and liver of rice frogs from both reference and contaminated site were dissected out, cut into small pieces, fixed in Davidson's fixative, processed by paraffin method. Tissue blocks were cut at 5 µm with a rotary microtome (Leica RM2165), collected on slides pretreated with Poly-L lysine overnight, after which the sections were de-waxed and hydrated. Unless otherwise stated, all incubations mentioned in what follows were performed at room temperature in a humid chamber, and all washing procedures consisted of three successive 5 min immersions in phosphate-buffered saline (PBS), pH 7.5. Endogenous peroxidase activity was blocked and melanin was bleached for 16 hrs. by incubation with fresh 3% H_2O_2, and after a rinse in PBS, antigens were exposed by heating by microwave for 5 min in 10 mM buffered Tris, pH 10.0. The sections were then washed again with PBS, treated with blocking reagent (IHC Select™) for 30 min to block nonspecific antibody binding, incubated 3 hrs. at 4 °C with the primary antibody against human FDCs (CNA-42) (DakoCytomation), washed with 0.05 % Tween-20 in PBS (PBS-T), and incubated for each 10 min with biotinylated secondary antibody and streptavidin HRP (IHC Select™). After further rinsing, the sections were finally developed using VIP® substrate working solution (Vector Laboratories),

immersed in deionized water to stop the reaction, counterstained with hematoxylin, dehydrated, and cover slipped. In each series of stained sections, positive and negative controls were included to assess the specificity of the assay. Sections of human tonsil were used as positive controls. Negative control slides were sections in which the primary antibody was replaced by *trp*E.

The result of immunostaining for human FDCs (CNA-42) in tissue samples showed that there was no specific stain against human FDCs (CNA-42) in any melanomacrophages and MMCs observed in spleen and liver of frog (Figure A-1and A-2).

Non-specific or unwanted specific staning of immunostaining for human FDCs (CNA-42) exhibited in spleen and liver of frog samples in this study might due to many factors such as antibody factors and tissue factors or might cause by a particularly strong positive charge of tissue.

For further study, the non-specific staining should be fixed to support to the hypothesis of a relationship exists between the MMCs of frog and the germinal centers of many birds and mammals.

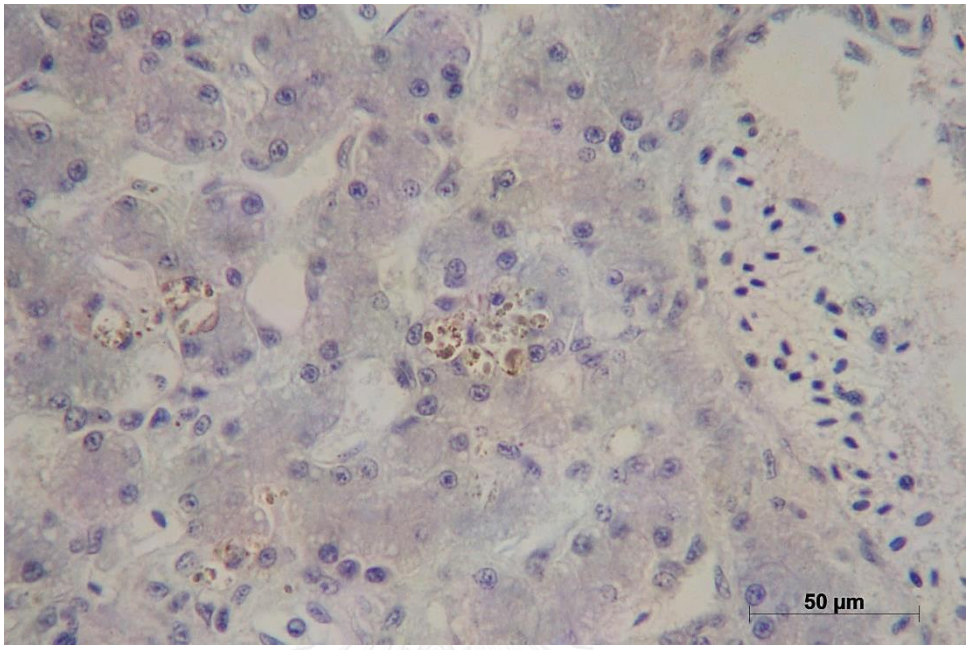

Figure A-1 Non-specific immunostaining for CNA-42 in liver of the rice frog, *Fejervarya limnocharis*, caught from agricultural area in Nan Province, Thailand.

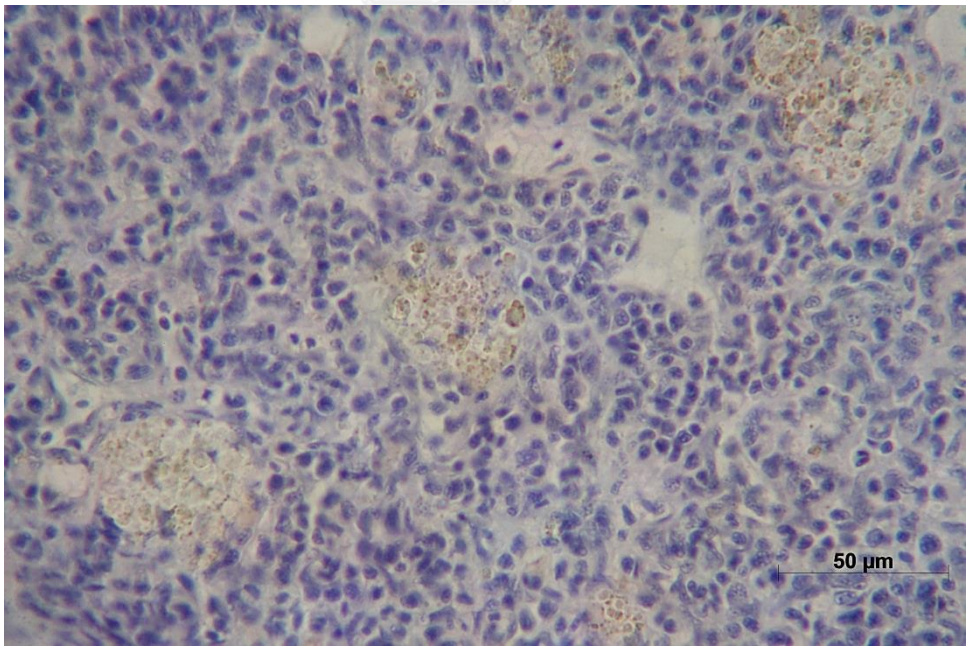

Figure A-2 Non-specific immunostaining for CNA-42 in spleen of the rice frog, *Fejervarya limnocharis*, caught from agricultural area in Nan Province, Thailand.

VITA

Mr. Khattapan Jantawongsri was born on March 17th, 1989 in Khon Kaen, Thailand. He has graduated a Bachelor of Science degree in Biology with first-class honors from Department of Biology, Faculty of Science, Khon Kaen University since 2011 with full support from the Development and Promotion of Science and technology Talents project (DPST). 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 agricultural areas and The Chulalongkorn University Forest and Research Station, Lainan Subdistrict, Wiang Sa District, Nan Province for more than a year as part of DPST and CU-ANR-56-04 grants support. During the last academic year of his study, he was a teaching assistant (General Biology Laboratory and Animal Physiology Laboratory) at Department of Biology, Faculty of Science, Chulalongkorn University. During his graduate practice, he has given both oral and poster presentations in the national conference entitled the 6th Conference for Science and Technology for Youths at Bangkok, Thailand in 2014. He has also presented part of his work as oral and poster presentations in the international conferences including the International Conference Environmental and Hazardous Substance Management (EHSM) at Bangkok, Thailand in 2013, the 7th Intercongress Symposium of the Asia and Oceania Society for Comparative Endocrinology (AOSCE) at Keelung, Taiwan in 2014, the 9th Society of Environmental Toxicology and Chemistry (SETAC) Asia/Pacific 2014 Conference at Adelaide, Australia in 2014, the Biodiversity & Health Conference, November at Phnom Penh, Cambodia in 2014 and the Vth International Wildlife Management Congress (IWMC) at Sapporo, Japan in 2015. In 2015, he has also published a part of his work as a research article in EnvironmentAsia, an international research journal listed in SCOPUS.