อิทธิพลของสารฆ่าวัชพืชต่อสัณฐานวิทยาและประชากรของกบหนอง *Fejervarya limnocharis* (Gravenhost, 1829) ในนาข้าว จังหวัดน่าน

ว่าที่ ร.ต. ภาณุพงศ์ ธรรมโชติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2555

INFLUENCE OF HERBICIDES ON MORPHOLOGY AND POPULATION OF RICE FROG *Fejervarya limnocharis* (GRAVENHOST, 1829) IN PADDY FIELDS, NAN PROVINCE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ภาณุพงศ์ ธรรมโชติ : อิทธิพลของสารฆ่าวัชพืชต่อสัณฐานวิทยาและประชากรของกบหนอง *Fejervarya limnocharis* (Gravenhost, 1829) ในนาข้าว จังหวัดน่าน . (INFLUENCE OF HERBICIDES ON MORPHOLOGY AND POPULATION OF RICE FROG *Fejervarya limnocharis* (GRAVENHOST, 1829) IN PADDY FIELDS, NAN PROVINCE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. นพดล กิตนะ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. วิเชฏฐ์ คนซื่อ, อ. ดร. จิรารัช กิตนะ,171 หน้า.

การใช้สารฆ่าวัชพืชในพื้นที่เกษตรกรรมสามารถทำให้เกิดการปนเปื้อนในสิ่งแวดล้อมและส่งผลกระทบต่อสิ่งมีชีวิตที่ไม่ใช่เป้าหมาย รวมถึงสัตว์สะเทินน้ำสะเทินบก จากการตรวจคัดกรองการปนเปื้อนสารฆ่าวัชพืช (แอทราซีน ไกลโฟเสต และ พาราควอต) ในนาข้าว จังหวัดน่าน พบว่ามีแอทราซีนปนเปื้อนในน้ำในนาข้าวที่มีการใช้สารฆ่าวัชพืช ในการศึกษานี้จึง ได้เลือกใช้กบหนอง *Fejervarya limnocharis* ที่อาศัยในพื้นที่เกษตรเป็นสิ่งมีชีวิตเฝ้าระวังเพื่อตรวจสอบอิทธิพลของสารฆ่าวัชพืชต่อสิ่งมีชีวิตที่ไม่ใช่เป้าหมายโดยเก็บตัวอย่างกบหนองจากนาข้าวที่มีการใช้สารฆ่าวัชพืช (พื้นที่ปนเปื้อน) และจากนาข้าวที่ไม่มีประวัติการใช้สารฆ่าวัชพืช (พื้นที่อ้างอิง) ผลการวิเคราะห์การตกค้างของสารฆ่าวัชพืชพบว่ามีสารฆ่าวัชพืชทั้งสามชนิดปนเปื้อนในกบจากทั้งสองพื้นที่ โดยที่พบพาราควอตในเนื้อเยื่อกบหนองจากพื้นที่ปนเปื้อนสูงกว่าอย่างมีนัยสำคัญ ผลการวิเคราะห์มอร์โฟเมตรี แกรวิเมตรี ของตับ ไต อวัยวะสืบพันธุ์ และ ตัวกบ พบว่ากบหนองในพื้นที่ปนเปื้อนมีค่าปัจจัยสุขภาวะต่ำกว่ากบหนองในพื้นที่อ้างอิงแสดงถึงอิทธิพลของสารฆ่าวัชพืชต่อสุขภาวะโดยรวมของกบหนอง ค่าดัชนีน้ำหนักตับของกบจากพื้นที่ปนเปื้อนมีค่าสูงกว่ากบจากพื้นที่อ้างอิงแสดงถึงการรับสารแปลกปลอมเข้าสู่ร่างกาย และยังพบว่าดัชนีน้ำหนักรังไข่ของกบหนองจากพื้นที่ปนเปื้อนมีค่าสูงกว่ากบหนองจากพื้นที่อ้างอิง ซึ่งอาจแสดงแนวโน้มการกระตุ้นการเติบโตของรังไข่โดยสารฆ่าวัชพืช เมื่อวิเคราะห์ความไม่สมมาตรทางกายวิภาคในกระดูกรยางค์ 4 ชิ้น ของกบหนอง พบว่ากบหนองจากพื้นที่ปนเปื้อนมีค่าความไม่สมมาตรทางกายวิภาคสูงกว่ากบหนองจากพื้นที่อ้างอิงอย่างมีนัยสำคัญแสดงถึงการได้รับสิ่งกระตุ้นให้เกิดความเครียดจากสิ่งแวดล้อมในช่วงการเจริญของกบ ผลความแตกต่างของสุขภาวะ น้ำหนักอวัยวะ และความไม่สมมาตรทางกายวิภาคของกบจากต่างพื้นที่แสดงให้เห็นว่าการใช้สารฆ่าวัชพืชสามารถสร้างผลลบต่อสิ่งมีชีวิตที่ไม่ใช่เป้าหมายได้หลายระดับ ข้อมูลจากการศึกษานี้อาจนำมาใช้เป็นสัญญาณเตือนถึงอันตรายของสารฆ่าวัชพืชต่อสุขภาวะของสัตว์มีกระดูกสันหลังชนิดอื่นที่อาศัยใกล้พื้นที่ที่มีการใช้สารฆ่าวัชพืชรวมทั้งมนุษย์

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สัตววิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : TISSUE RESIDUE / SENTINEL SPECIES / AMPHIBIAN / MORPHOMETRY / GRAVIMETRY / FLUCTUATING ASYMMETRY

PANUPONG THAMMACHOTI : INFLUENCE OF HERBICIDES ON MORPHOLOGY AND POPULATION OF RICE FROG *Fejevarya limnocharis* (GRAVENHOST, 1829) IN PADDY FIELDS, NAN PROVINCE. ADVISOR : NOPPADON KITANA, Ph.D., CO-ADVISOR : ASST. PROF. WICHASE KHONSUE, Dr. Hum. Env., JIRARACH KITANA, Ph.D., 171 pp.

Herbicide utilization in agricultural area can lead to an environmental contamination and adverse effects on non-target organisms including amphibians. Screening for herbicide contamination (atrazine, glyphosate and paraquat) in paddy fields of Nan Province showed contamination of atrazine in water at the field with intensive herbicide utilization. The rice frog *Fejervarya limnocharis* living in agricultural area was thus used as a sentinel species to test for herbicide influence on non-target organisms. Frogs were field collected from a paddy field with intensive herbicide usage (a potential contaminated site) and a reference paddy field with no history of herbicide usage. Results of herbicide tissue residue analysis showed that detectable levels of these three herbicides were found in frogs from both sites with a significantly higher level of paraquat in the contaminated site animals. Results of morphometry and gravimetry of liver, kidney, gonad and body showed that frogs from the contaminated site had a significantly lower condition factor indicating potential impact on overall health of the frog, a significantly higher liver weight indicating potential exposure to xenobiotics detoxification, and a significant increase in ovarian weight compared to those of the reference site possibly due to effects of herbicide on ovarian growth. The results of fluctuating asymmetry analysis of four appendage bones showed that FAs of frogs from the contaminated site were significantly higher than those of the reference site indicating exposure to environmental stressor during developmental process of frog. Overall, site-related differences in health status, gravimetric parameters and fluctuating asymmetry indicate that herbicide utilization could pose adverse effects at different levels to this non-target organism. These results could be used as an early warning of environmental health hazards for other vertebrates living near the herbicide utilization area, including human.

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Field of Study : Zoology Advisor's Signature .....
Academic Year : 2012 Co-advisor's Signature .....
Co-advisor's Signature .....

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I would like to dedicate merits of my thesis to the rice frogs that were sacrificed in this study. May their souls rest in peace.

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LIST OF ABBREVIATIONS

B.C.	British Columbia
BW	body weight
°C	degree Celsius
CA	California
CF	Condition factor
cm	centimeter
DA	Directional asymmetry
DI	Developmental instability
etc.	et cetera (and so on/and so forth)
F	Female
FA	Fluctuating asymmetry
FL	Florida
g	gram
x g	time the strength of earth gravitational field
GSI	gonadosomatic index
HSI	hepatosomatic index
kg	kilogram
L	liter
LOEL	lowest-observed-effects level
LOEC	lowest-observed-effects concentration
M	Male
m	meter

MA	Massachusetts
MD	Maryland
mg	milligram
MI	Michigan
min	minute
mL	milliliter
mm	millimeter
MS-222	Ethyl 3-aminobenzoate methanesulfonic acid
N/A	not available
ng	nanogram
NH	New Hampshire
NOEL	no-observable-effect level
NOEC	no-observable-effect concentration
ppb	part per billion (µg/L or µg/kg or ng/g)
ppm	part per million (mg/L or mg/kg or µg/g)
RSI	renosomatic index
sec	second
SVL	snout-vent length
UV	Ultra-violet radiation
µg	microgram
µL	microliter
µm	micron, micrometer
%	percentage

CHAPTER I

INTRODUCTION

Currently, environmental contamination has become one of the global environmental problems occurring in all over the world. Sources of contamination include both industrial and agricultural activities. In agriculture, use of agrochemicals, especially pesticides, is regarded as one of the prominent means of environmental contamination due to a limit capability of agrochemicals to be degraded in nature (Plimmer, 2001). When the pesticides were used intensively and continuously for a long time, their residues are likely to be present in the agricultural areas. Non-target organisms living in these areas are thus susceptible to an exposure with the pesticide residues and an adverse effect from this contamination (Hughes, 1996).

Pesticide contamination may affect on any vertebrates living in vicinity of the agricultural areas including human (National Research Coucil: NRC, 1991). Therefore it is essential to monitor the extent of contamination in physical environment as well as in a representative vertebrate living in the affected area. Also, since non-human vertebrates share several similar structures and functions of their organs to those of human, monitoring adverse changes in the animal life could be used as an early warning of potential impacts from pesticide contamination to human, making it a sentinel species for environmental health hazards (NRC, 1991).

According to the NRC (1991), the sentinel species should have 1) a measurable response to the agents in question, 2) a territory or home range in the monitored area, 3) a sufficient population size and density to permit enumeration and 4) should be easily enumerated and captured. Amphibian is regarded as one of a suitable sentinel species for environmental contamination. Their natural habitats include agricultural areas where they can be exposed to pesticides (Khan and Law, 2005). Their existence both in water and on land makes them susceptible to pesticide exposure through several routes, especially through their semi-permeable skin (Duellman and Trueb, 1994; Roy, 2002; Quaranta et al., 2009).

Previous studies showed that pesticides can affect many vital functions of amphibian life such as growth and reproduction, and may also pose serious threat at

population level (Govindarajuru, 2008 and Solomon et al., 2008). Studies on morphology and population of amphibian can thus be used to evaluate long-term impact of pesticide contamination on vertebrates (Ouellet M, 2000; Du Preez et al., 2005; Soderman et al., 2007). In addition, since amphibian decline has become a global problem for a few decades and the environmental contamination is regarded as the main 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, climate change, infection, habitat destruction, or synergistic of these factors (Brige et al., 2000).

In Thailand, especially Nan Province in the north, there are a lot of agricultural areas where pesticides have been used for a long time. Most of pesticides used in this area include herbicides namely atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), glyphosate (N-(phosphonomethyl) glycine) and paraquat (1,1'-dimethyl-4,4'-bipyridinium ion). Atrazine is a known endocrine-disrupting chemicals that can affect on reproductive system of amphibians (Kavlock, 2001; Hayes et al., 2002, 2006, 2010; Kloas and Lutz, 2006), while glyphosate and paraquat are also found to show similar adverse effect on reproductive system and lethal impact of amphibians (Relyea, 2005; Quassinti et al., 2009). 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. Therefore, it is of importance to examine whether utilization of these herbicides will have any impact on the amphibians living in these areas. Adverse effect found in the rice frog could be used as an early warning of environmental health problems for other vertebrates living near agricultural areas including human.

Approach of the Study

Herbicides including atrazine, glyphosate and paraquat have been used in paddy fields at Nan Province for a long time. It is possible that these herbicides can contaminate in paddy fields and affect non-target organisms living in these areas. In this study, the rice frog *Fejervarya limnocharis* was used as a sentinel species of

environmental health hazards from the herbicide contamination since stable population of *F. limnocharis* can be found in the agricultural areas making it susceptible to long term exposure and accumulation of xenobiotics (Othman et al., 2009). The rice frog was collected from the paddy fields with different degree of herbicide utilization and subjected to examination for multiple parameters in difference level of biological organization from tissue to population level (Figure 1.1). Herbicide tissue residue analysis was performed to examine an effect at tissue level. Morphomertry and gravimetry of liver, kidney and gonad was carried out to examine effects at organ level. Condition factor or an indicative of overall health was examined to determine an effect at organismal level. In addition, the populated frog was analyzed for fluctuating asymmetry, and age structures to examine for effects at population level. Association between herbicide residues, morphometric and gravimetric parameters were examined in order to test for influence of herbicide contamination on non-target organisms.

Objectives

1. To examine herbicide contamination in environmental samples and tissues of the rice frog living in contaminated and reference paddy fields
2. To compare morphology, anatomy and population parameters of the rice frog living in contaminated and reference paddy fields
3. To determine association between herbicide contamination and morphology and population of the rice frog living in paddy fields

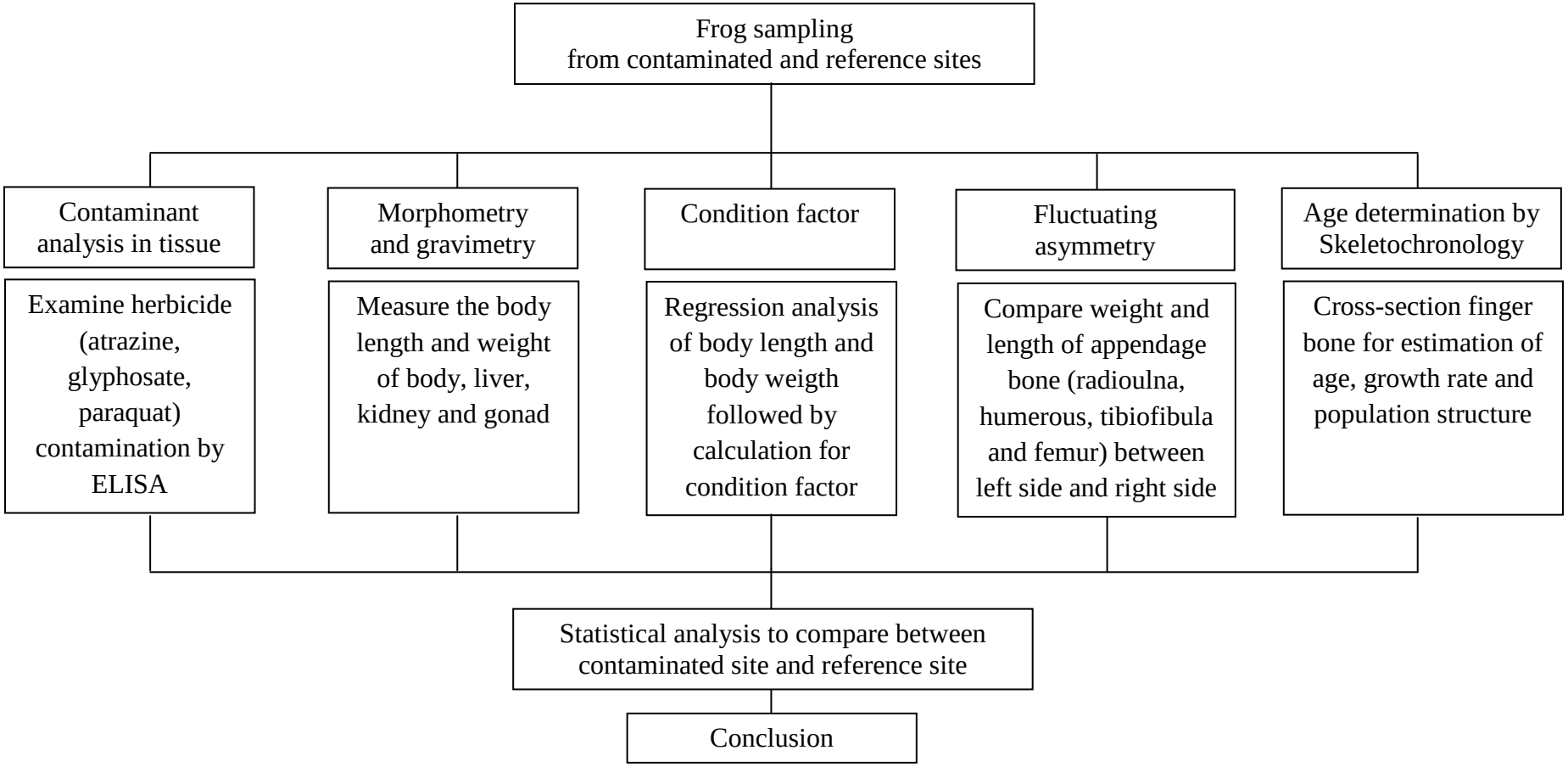


Figure 1.1 Research scope of this study

CHAPTER II

LITERATURE REVIEWS

1. Agricultural activities in Nan Province

Nan Province is located in the eastern part of northern region of Thailand. Nan Province has a total area of 11,472 km^2 with population of more than 475,000 individuals. People of Nan Province have been a farmer for a long time, 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 were paddy rice and backyard garden (Thadaniti and Prajuabmoh, 2005). In Wiang Sa District, the central-southern part of Nan Province, more than 96.7 % of the land is used for agricultural purposes (Wiang Sa District Agricultural Extension Office [DAEO], 2012)

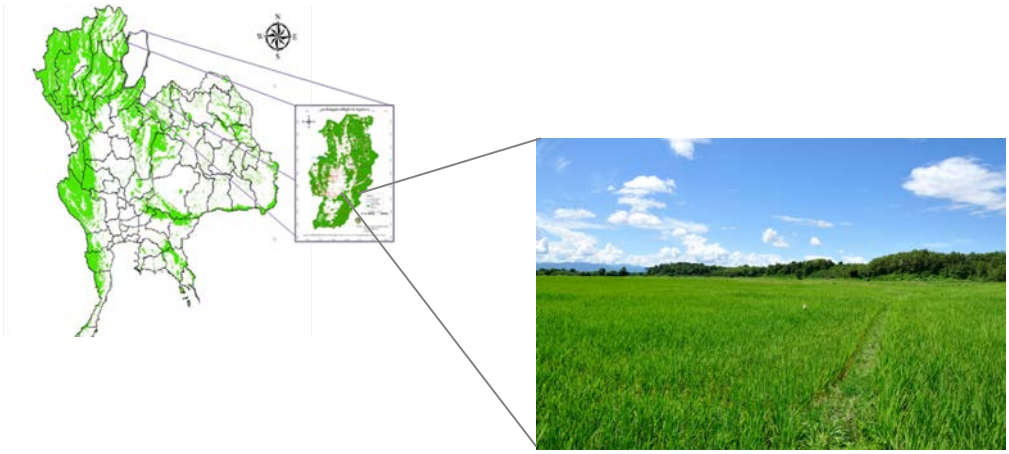


Figure 2.1 Agricultural areas in Nan Province, northern part of Thailand (modified from Forest Land Management Office website: http://www.forest.go.th/fl_mgt/index.php)

At present, agricultures in Nan Province are diversified to include maize, tobacco, rubber tree, soy bean, orange and litchi orchards in addition to the paddy rice (Wiang Sa DAEO, 2012). These agricultural activities involve an intentive use ofagrochemicals such as pesticides and fertilizers for a long time. The report of Nan Provincial Agricultural Extension Office (PAEO) in 2008 showed that the amount of

agrochemicals imported to Nan Province was 1,274,100 kg at the value of 232.1 million Baht. Among these chemicals, more than 92.04% was herbicides (1,172,700 kg), followed by chemicals for plant diseases 4.44% (56,600 kg) and insecticides 3.52% (44,800 kg) (Janphong, 2008; Figure 2.2).

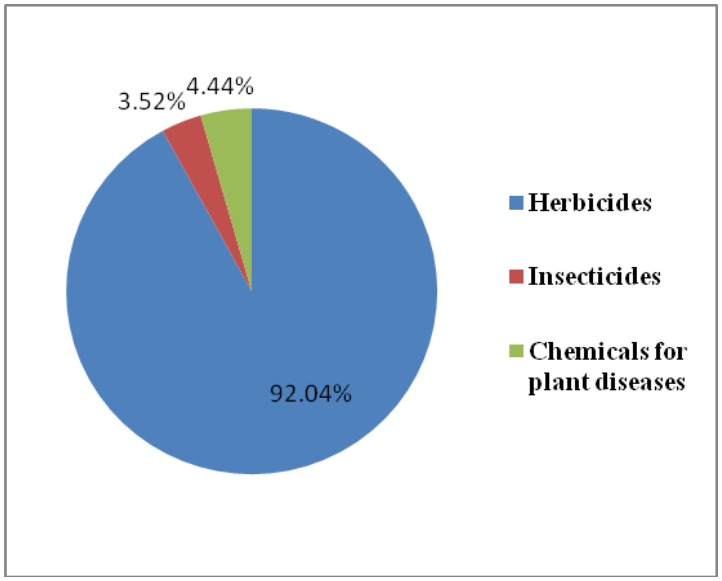


Figure 2.2 Percentage of pesticide categories imported into Nan Province in 2008 (Janphong, 2008)

In the paddy fields, especially, most of pesticides widely used in Nan Province were herbicides. The prevalent herbicides used in the paddy fields were glyphosate and paraquat (Panuwet et al. 2012 and Wongwichit et al., 2012). In addition, during a field survey in Nan Province, atrazine utilization in the paddy field was also evidenced in addition to glyphosate and paraquat. These herbicides have been intensively used as herbicide-mixture in the same area or used at different period depending on yearly crop cycle.

2. Herbicides

2.1 Atrazine

2.1.1 Atrazine Background

Atrazine (6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4diamine) 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). Now adays, atrazine is the most generally used herbicide in the USA (Hayes et al., 2002). However, atrazine is known as the endocrine disrupting chemicals (EDCs) that can exert its effect on animals or the non target organisms (Deb, 2005 and Hayes et al., 2006). US 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 (US EPA, 2009)

2.1.2 Environmental Fate of Atrazine

In terrestrial habitat, atrazine is not rapidly degraded on leaf of plant because atrazine is resistant to abiotic hydrolysis. The most important route of degradation is microbial decomposition in oxygenated conditions. Atrazine reasonably are susceptible to degradation in soil. The half-life in soil is about 3-4 months (US EPA, 2003b). Moreover, the relatively low adsorption in soil of atrazine indicates that it can leach from soil to surface and ground water. Degradation products of atrazine in soil include 2-chloro-4-amino-6-isopropylamino-1,3,5-triazine, 2-chloro-4-ethylamino-6-amino-1,3,5-triazine, 2-chloro-4-amino-6-amino-1,3,5-triazine, 2-hydroxy-4-ethylamino-6-isopropylamino-1,3,5-triazine, and the main metabolite, 2-hydroxy-4-amino-6-isopropylamino-1,3,5-triazine (WHO, 1996). In addition, Suvanakhetnikhom (2004) reviewed that the half-life of atrazine in Thailand environment is more than 60 days and can contaminate for upto 7-8 months in soil at agricultural area.

In aquatic habitat, atrazine is a persistent and mobile substance in environment and present in both surface and groundwater. The s-triazine ring make atrazine resistant to microbial degradation in aquatic systems (Howard, 1991). It is also resistant to direct aqueous photolysis and abiotic hydrolysis. As a result, the half-life was much longer than in soil condition. In an aerobic aquatic environment, the half-life in water is 578 days and in sediment is 330 days. The half-life also depends on climatic condition, as atrazine is more persistent in colder climate (US EPA, 2003b). In water, major degradation products of atrazine including hydroxyatrazine and

desethylatrazine have been detected frequently in ground water at low levels (Plimmer, 2001). The relative persistence of atrazine in surface water increases potential for exposure in aquatic organisms, particulary those in static water systems (Solomon et al., 2008).

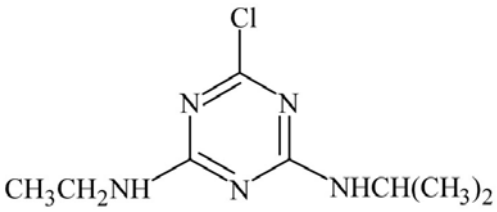


Figure 2.3 Chemical structure of atrazine

2.1.3 Effect of Atrazine on Amphibians

In the past, atrazine was belived to be toxic to amphibians only in high concentration. However, conflicting evidences have emerged during the past 10 years. Hayes et al. (2002) reported that atrazine can affect on reproductive and development of frog even at the low dose. He proposed that atrazine should be regarded as an endrocrine disrupting chemicals that exerts its effects by inducing aromatase activity, an enzyme responsible for convesion of testoseterone to estrogen in vertebrates. Hayes et al. (2002) found that exposure to low level of atrazine (0.01 ppb) caused male *Xenopus leavis* tadpoles to turn into hermaphrodrite frog as revealed by the gonadal histology and higher dose (1 ppb) could cause demasculinization by reducing the laryngeal size. After that, Hayes et al. (2003) also reported that atrazine could feminize male northern leopard frog *Rana pipiens* at low concentrations (0.02-6.7 ppb) even in natural population. In 2006, Hayes et al. also reported that atrazine (0.1µg/L) in combination with other pesticide mixture or atrazine in commercially use formulation can impact on larval growth and development of the leopard frog (Hayes et al., 2006). In the recent years, Hayes et al. (2010) also reported that atrazine could change 75% of male frogs *Xenopus leavis* to become sterile and turned one in ten male frogs into female frogs.

Meanwhile, other researchers also confirmed the impact of atrazine on amphibians. The researchers from Tufts University reported in 2008 that the morphological deformity of internal organs (heart, kidney and digestive system) was found in *Xenopus leavis* tadpoles exposed to 25-35 mg/L of atrazine. In addition, Solomon et al. (2008) reviewed and showed that a number of reports was available on effects of atrazine on aquatic vertebrates, especially amphibians. At present, Bishop et al. (2010) mentioned that atrazine is an important toxic herbicides that can greatly affect amphibians since atrazine use was found in agricultural area where amphibian lived and exposed during egg, laval development periods as well as chronically throughout the year to adults. They also reviewed that atrazine and other herbicide mixtures could cause malformation and edema, increase incidence of parasite and disease, impact on metamorphosis growth and development in amphibian larvae.

2.2 Glyphosate

2.2.1 Glyphosate Background

Glyphosate (N-phosphonomethyl glycine) is an herbicide with trade names as Round up, Rodeo, Shackle, etc. Glyphosate is the most commonly used non-selective broad-spectrum and post-emergence herbicides in the world (Govindarajulu, 2008). Glyphosate is broadly used in agricultural acitivities to suppress annual and perennial weeds. In Thailand, glyphosate herbicide was the first rank of imported herbicide in recent years (Office of Agriculture Regulation; OAR, 2012). Glyphosate is a weak organic acid comprising of a glycine moiety and a phosphonomethyl moeity. Glyphosate prevent the synthesis of essential aromatic amino acids in plants and some microorganisms by inhibiting the enzyme 5-enolpyruvyl shikimate-3-P synthetase (Devine et al., 1993). 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 (US EPA, 2009).

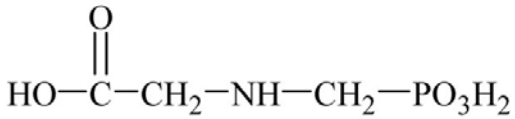


Figure 2.4 Chemical structure of glyphosate

2.2.2 Environmental Fate of Glyphosate

In terrestrial habitat, glyphosate absorbs strongly to soil and would not move below 6 inch of soil layer (US EPA., 1993). Glyphosate is an organophosphorus compound (carbon-phosphorus bond), causing it to be resistant to hydrolysis, thermodecomposition and photolysis (Moore et al., 1983). Several reports discussed that the photochemical decomposition is not a pathway of degradation for glyphosate in soil. But Lund-Hoie and Friedstad (1986) discussed that glyphosate may degrade by photolysis. Nontheless, glyphosate can be degraded by soil microorganisms which use it as a source of carbon, nitrogen and phosphorus (Petit et al., 1995).

In aquatic habitat, glyphosate might be degraded by microbes. Even though glyphosate is highly water soluble, it appears to have a low potential to move to ground water due to their strong absorbtive characteristics. However, glyphosate does have the potential to contaminate in surface waters. If glyphosate were to reach surface water, it would be resistant to hydrolysis and aqueous photolysis (US EPA., 1993).

2.2.3 Effect of Glyphosate on Amphibians

Howe et al. (2004) reported that tadpoles of the northern leopard frog (*Rana pipiens*) showed decreased in body length at metamorphosis, increased time to metamorphosis, present with tail and gonad abnormalities upon chronically exposure to the glyphosate (0.6 and 1.8 mg formulation acid equivalents/L). In addition, they also reported that glyphosate surfactant (polyethoxylated tallowamine surfactant; POEA) also affect on growth and development of frog. For the effect of glyphosate on adult amphibians, Relyea (2005) indicated that survival rate of frogs was decrease when they were sprayed with 1.6 ppm of glyphphosate. Moreover, Govindarajulu (2008) also reviewed that glyphosate can impact on amphibians directly and indirectly through several routes. The direct impact includes reducing survival rate, developmental abnamalities, change in behaviors and also genomic damage, while the indirect impact includes interaction with predators and competitive stress. It was also suggested that physical factors such as temperature, UV radiation, pH and soil could cause synergistic adverse effects with glyphosate in natural condition.

2.3 Paraquat

2.3.1 Paraquat Background

Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) is one of the most extensively used herbicides in the world (Eisler, 1990). Paraquat is a broad spectrum contact weed killer and herbage desiccant that is commonly used in agriculture. Paraquat is used for pre-emergence crops and also used for controlling aquatic weeds. Paraquat adhere to plant surface exerts its effect by inhibiting the process of photosynthesis and respiration (Haley, 1979). Mode of action of paraquat in plant includes lipid peroxidat ion of membranes owing to superoxide radicals which is similar to the processes in animals (Summers, 1980). At present the maximum contamination level of paraqut is not determined. But for diquat, a chemical in the same herbicidal group, the contaminant level is set at 0.02 mg/L in drinking water (US EPA, 2009).

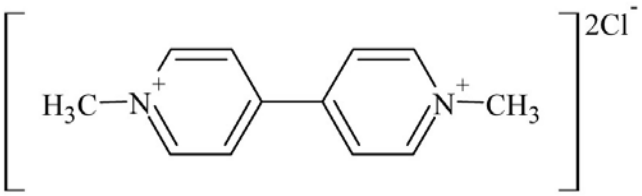


Figure 2.5 Chemical structure of paraquat

2.3.2 Environmental Fate of Paraquat

In terrestrial habitat, paraquat binds strongly to soil particles and tends to remain strongly bound for a long time in an inactive state, even though it can be desorbed and become biologically active in some situations. These result from their cationic properties, especially with clay that has a cation-exchange capacity. Half-life of paraquat in soil can be as long as 20 years (Watts, 2011). Paraquat can be photolysis in soil by UV radiation in the ozone or oxygenated condition. Due to their unavailability when they are adsorbed to the soil particles, the biodegradation are relatively reduced (Petit et al., 1995).

In aquatic habitat, paraquat has been found in surface waters, drinking water, and in groundwater, although it is believed to be immobile in the soil and not to leak to groundwater. Albeits its strong adsorption to particles and sediment, paraquat is

rapidly photodegraded in freshwater by UV light and also biodegraded by aquatic microorganisms. Watts (2011) reviewed that a half-life of paraquat was estimated to be between 2 and 820 years depending on sunlight and depth of water.

2.3.3 Effect of Paraquat on Amphibians

Dial and Bauer (1984) indicated that 0.1 ppm of paraquat can affect on growth and development of early developmental stage of the northern leopard frog (*Rana pipiens*) and also effect on swimming behavior of the tadpole. In addition, at 5 ppm, paraquat can affect on morphological abnormality and reduce growth rate of early developmental stage of tadpole. Dial and Dial (1987) also found that 2 ppm of paraquat exposed to the northern lepard tadpole showed the growth retardation and increased in developmetal abnormalities (tail malformation and head defects). Eisler (1990) also reviewed that paraquat can affect on aquatic organisms including amphibian. He reported that at 500 ppb (0.5 ppm) of paraquat, it can affect on survival rate, and developmental abnormalities of tadpoles.

Table 2.1 Summary of chemical properties and toxicities of atrazine, glyphosate and paraquat

Property/Toxicity	**Atrazine**	**Glyphosate**	**Paraquat**
Compound	Triazines [(1)]	Organophosphorous [(2)]	Bipyridylium [(2)]
Empirical formula	$C_8H_{14}ClN_5$ [(1)]	$C_3H_8NO_5P$ [(3)]	$C_{12}H_{14}Cl_2N_2$ [(4)]
CAS registry number	1912-24-9 [(1)]	38641-94-0 [(3)]	1910-42-5 [(3)]
Melting point	172-175 °C [(5)]	200 °C [(5)]	175-180 °C [(2)]
Solubility in water	33 mg/L at 25°C [(1)]	12 g/L [(2)]	561 g/L [(2)]
Half-life in terrestrial habitat	13-261 days [(1)]	60 days [(5)]	> 10 years [(2)] (in field studies)
Half-life in aquatic habitat	41-237 days [(10)]	7 days in silty clay loam sediments [(3)]	< 2 weeks [(3)]

Table 2.1 Summary of chemical properties and toxicities of atrazine, glyphosate and paraquat (continued)

Property/Toxicity	**Atrazine**	**Glyphosate**	**Paraquat**
Acute toxicity - LD_{50} in rat (oral)	1,869-3,080 mg/kg [1]	4,320 mg/kg[11]	95-174 mg/kg [12]
Acute toxicity to fish - LC_{50} in fish (96 hr)	>100 mg/L [10] (Carp)	86 mg/L [5] (Rainbow trout)	15-32 mg/L[5] (Rainbow trout)
Acute toxicity to amphibian - LC_{50} in amphibian	> 48 mg/L (*Bufo americanus*, 8 days) [10] 410 µg/L (*Rana catesbeiana*, 8 days) [10]	49.4 mg /L (*Crinia insignifira*, 48 hr) [9] 4.25-11.47 mg /L (*Rana pipiens*, 96 hr) [9]	100 mg/L (*Limondynastes peronii*, 96 hr) [12] 262 mg/L (*Adelotus brevis*, 96 hr) [12]
Chronic toxicity - NOEC/NOEL	0.2 mg/L (*Rana pipiens*; 138 days on growth) 0.8 mg/L (*Xenopus laevis*; 35 days on lethality) [10]	362 mg/kg/day in male rat [11] 457 mg/kg/day in female rat [11]	≤ 1 mg/L in aquatic vertebrates [12]
Chronic toxicity - LOEC/LOEL	0.8 mg/L (*Xenopus laevis*; 35 days on lethality) [10]	940 mg/kg/day in male rat [11] 1,183 mg/kg/day in female rat [11]	0.5 mg/L in aquatic sensitive organisms [12]
Maximum contaminant level for drinking water	0.003 mg/L [6]	0.7 mg/L [6]	-
Maximum residue limit in food	0.04 mg/kg (meat and cattle) [7]	0.05 mg/kg (poultry meat) [8]	0.005 mg/kg (poultry meat) [8]
Ecological risk concentration level	0.01-0.02 mg/L [1]	-	-

Remarks:

- (1) US EPA, 2003b; (2) Petit et al., 1995; (3) US EPA, 1993; (4) US EPA,1997; (5) Petit et al., 1995; (6) US EPA, 2009; (7) Health Canada, 2010; (8) Codex Alimentarius, 2006; (9) Govindarajulu, 2008; (10) Solomon et al. 2008; (11) Miller et al., 2010; (12) Eisler, 1990

- NOEC = no-observable-effect concentration, NOEL = no-observable-effect level, LOEC = lowest-observed-effects concentration, LOEL= lowest-observed-effects level

3. Amphibians As a Sentinel Species

A sentinel species is an organism that has been used by scientists to determine the ecological risk assessment and monitoring the environment. According to van der Schalie et al. (1999), the non human organism can react to an environmental contamination before the impacts could occur to human. The sentinel species can offer the possibility of expanding our understanding and response to the environmental health concerns. Jamil (2001) commented that sentinels were regarded as biomonitors by acting as the early alarm signals. The using of animals as a sentinel species has several merits including;

1. Sentinel species may provide early warnings of potential risks before the risks break out in human.
2. In some toxicant, the toxic effect are similar in sentinel species and human
3. The condition of exposure may be comparable under some situation, such as non-target organisms and people.

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.

Loumboudis et al. (1999) and Roy (2002) mentioned that amphibian should be regarded as a good environmental sentinel species for chemical contamination. Amphibians show several specials characters that support them as good indicators of pollution due to their semi-permeable skin, living in both aquatic (tadpole) and terrestrial (adult frog), allowing exposure to the pollutant in water or soil during of the whole life cycle. In addition, as a member of vertebrates, the effect occurred in amphibian could also occur to other vertebrates including human.

4. Effect of Pesticides on Morphology and Population of Amphibians

Previous studies have been shown that herbicide contamination can affect on morphology and population of amphibians such as amphibian deformities as a result of pesticide contamination (Ouellet, 2000). Pesticide utilization, 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 reproductive and developmental systems as well as reduced survival rate of the population. Even with a small amount of endocrine disrupting chemicals (EDCs) exposure, it could cause adverse health effect on these animals in a long run.

In contradiction, Du Preez et al. (2005) reported that effect of atrazine on population of African clawed frog *Xenopus laevis* living in corn field was minimal with no significant difference in age structure of frog living in reference and contaminated sites. Solomon et al. (2008) also reported that, using African bull frog *Rana catesbeiana* as a subject, there was no relationship between a number of population, reproductive parameters and atrazine concentration in ponds.

Hayes et al. (2006) studied the effect of pesticides on *Rana pipiens* and found that both single (such as atrazine) and mixture of pesticides can significantly reduce body weight and length of frog, indicating that pesticides can affect on morphology of frog living in an agricultural area. McCoy et al. (2008) 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.

In other chemical contamination, Soderman (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. Othman (2009) studied an effect of cadmium contamination on rice frog *Fejervarya linocharis* living in paddy fields and found that cadmium could accumulate in frog tissues, and the affected frogs showed changes in morphology of liver, kidney and gonad.

5. Fluctuating Asymmetry as Indicator for Environmental Stress on Amphibian

During the developmental process of organism, an ideal perfect symmetry is regarded as the reflection of developmental stability (Zakharov, 1992). However, the reduced in developmental stability could probably lead to deviation from this perfect symmetry. The random deviation from perfect symmetry is generally known as a fluctuating asymmetry (FA; Palmer and Strobeck, 1986) which is usually used as a measure of developmental stability in several organisms. Factors effecting on developmental pattern of organisms and leading to fluctuating asymmetry (Palmer, 1994) include;

Developmental stability is a natural process occurred to defense or oppose the developmental disruption. The developmental stability includes homeostatic mechanism in animal body; negative feedback of enzyme activity or the nervous control of the non-adjacent structure.

Developmental instability (noise) is a process occurred to disrupt the normal development of organism such as environmental stress or genetic problem leading to a small random difference in rate of cell division, growth, and morphological change of the cell.

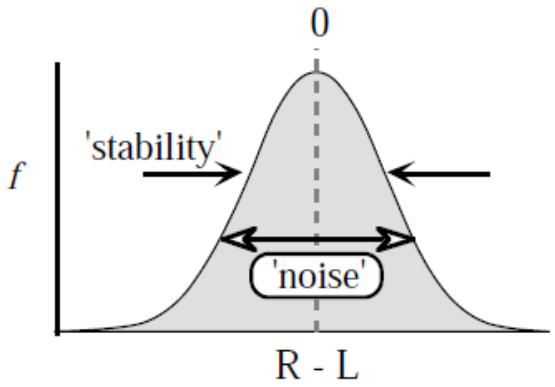


Figure 2.6 The pattern of FA reflects the cooperation between two contrasting process: Developmental stability and developmental instability (noise) (Palmer, 1994)

The developmental instability (noise) may come from environmental stress (Graham et al., 2010). According to Escos et al., (2000), they define stress as any environmental factors that cause a reduction in the efficient use of energy, causing a reduction in developmental homeostasis, and finally reducing long term total inclusive fitness. Valentine et al. (1973) mentioned that the fluctuating asymmetry analysis is an effective indicator of environmental stress in fish.

Lauck (2006) discussed that environmental stress induced by logging activity could increase the fluctuating asymmetry in the amphibian. Similarly, Soderman et al. (2007) mentioned that environmental stress induced by acidifed habitat can disrupt the developmental stability and increase the fluctuating asymmetry of amphibians.

6. Skeletochronology for Age Determination of Amphibian

The age of animals is an important parameter to understand their life history. An accurate age determination is regarded as an essential basis for population ecology and developmental biology of animals including the amphibians (Smirina, 1994). Knowing the age could lead to addition crucial information of amphibians life history such as age at maturity, age at metamorphosis, longevity and growth rate. Therefore, several methods including mark and recapture, extrapolation from size-frequency data, morphological examination of gonad and skeletochornology have been developed and applied for amphibians study (Halliday and Verrell, 1988).

At present, skeletochronology is regarded as a well definced method widely accepted for determination age of the amphibians (Halliday and Verrell, 1998)**.** The skeletochronology is the method that determines the age from number of line of arrested growth (LAGs) formed in long bones as a result of environmental fluctuation within a year. In amphibians living in the clear seasonality such as the temperate regions or the in the dessert area, the LAGs was formed during the hibernation or aestivation periods respectively. A few studies have attempted, with varied degree of success, to apply this technique with amphibians in tropical zone where the seasonality is not clear (Khonsue et al., 2000). Although the skeletochronology is consistency, the limitation of this method (Castanet and Smirina, 1990) includes;

1. Presence of line of metamorphosis (one form of LAG) could occur after metamorphosis from the tadpole and could cause some confusion with other LAGs.
2. False line might occurred when more than one line was present in one year
3. Resorption and remodeling line resulted from decomposing of bone near bone narrow could cause evanescent of the LAGs
4. Intensity of line depends on external environment. In the area with clear seasonality, LAG intensity is relatively stronger that those in the unclear seasonal area.

7. The Rice Frog

Figure 2.7 The rice frog *Fejervarya limnocharis* (Gravenhost, 1829)

The rice frog *Fejervarya limnocharis* (Gravenhost, 1829) is also known as Asian grass frog, common pond frog, field frog, grass frog and Indian rice frog. This species is regarded as one of the cryptic amphibian species (Zug et al., 2001). The assessment information of the IUCN Red List Category & Criteria is identified it as the "least concern" status meaning that the rice frog is widely distributed, tolerant to a broad range of habitats, and its population is presumably large and appears to be stable at present.

The rice frog is the small amphibian which has the total length of approximately 42-46 milliliters (Chan-ard, 2003). The external morphology for description is as follow. 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. Special characters in male frog is the throat which is mottled with brown (Taylor, 1962 and Amphibia Web, 2012).

Taylor (1962) 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 many kind of snakes.

The classification of the rice frog is as followed:

Kingdom Animalia

Phylum Chordata

Subphylum Vertebrata

Class Amphibia

Superorder Salientia

Order Anura

Family Dicroglossidae

Genus *Fejervarya*

Species *Fejervarya limnocharis* (Gravenhost, 1829)

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

Figure 2.8 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)

In the past, there were two 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 (Othman, 2009).

Wu et al. (2012) studied on the potential toxic effects and contamination of DDT and its metabolites on rice frog from paddy fields in South China and found that DDT can be accumulated in the rice frog, especially in liver and egg of the rice frog. Further analyses for an impact of these contaminations on the rice frog were recommended.

CHAPTER III

HERBICIDE CONTAMINATION IN ENVIRONMENT AND TISSUE OF RICE FROG *Fejervarya limnocharis* LIVING IN PADDY FIELDS AT NAN PROVINCE

Introductions

In Thailand, especially Nan Province in the north, there are a lot of agricultural areas where pesticides have been used. Most of pesticides used in this area include herbicides namely atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), glyphosate (N-(phosphonomethyl) glycine) and paraquat (1,1'-dimethyl-4,4'-bipyridinium ion). When these herbicides were used intensively and continuously for a long time, their residues are likely to be present in the agricultural areas and it may affect on any vertebrates living in vicinity of the agricultural areas including human. Therefore, it is essential to monitor the extent of contamination in physical environment as well as in a representative vertebrate living in the affected area. Also, since non-human vertebrates share several similar structures and function of their organs to those of human, monitoring adverse changes in the animal life could be used as an early warnings of potential impacts from pesticide contamination to human, making it a sentinel species for environmental health hazards (NRC, 1991).

Amphibians are regarded as one of a suitable sentinel species for environmental contamination. Their natural habitats include agricultural areas where they can be exposed to pesticides (Khan and Law, 2005). Their existence both in water and on land makes them susceptible to pesticide exposure through several routes, especially through their semi-permeable skin (Duellman and Trueb, 1994; Roy, 2002; Quaranta et al., 2009).

Atrazine is a known endocrine-disrupting chemicals that can affect on reproductive system of amphibians (Kavlock, 2001; Hayes et al., 2002, 2006, 2010; Kloas and Lutz, 2006) while glyphosate and paraquat are also found to show similar adverse effect on amphibians (Relyea, 2005; Quassinti et al., 2009). 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. Therefore, it is

interesting to examine whether the herbicide will be able to contaminate in amphibian living in agricultural area.

In this study, herbicide residues were analyzed in environmental samples in order to examine the extent of contamination in agricultural and related areas. Then, the rice frog *Fejervarya limnocharis* was used as a sentinel species of environmental health hazards from the herbicide contamination since stable population of *F. limnocharis* can be found in the agriculture areas making it susceptible to long term exposure and accumulation of xenobiotics (Othman et al., 2009). The rice frog was collected from the paddy fields with different degree of herbicide utilization and subjected for herbicide residue analysis.

Objective

To examine herbicide contamination in environmental samples and tissue of the rice frog *Fejervarya linocharis* living in paddy fields at Nan Province.

Materials and Methods

1. Study Sites

In Nan Province, there are many agricultural areas where herbicides have been used for along time, especially in Wiang Sa District where a lot of paddy fields with intensively use of herbicides are present. Therefore, the two paddy fields in Wiang Sa District, Nan Province were considered to be the study sites including:

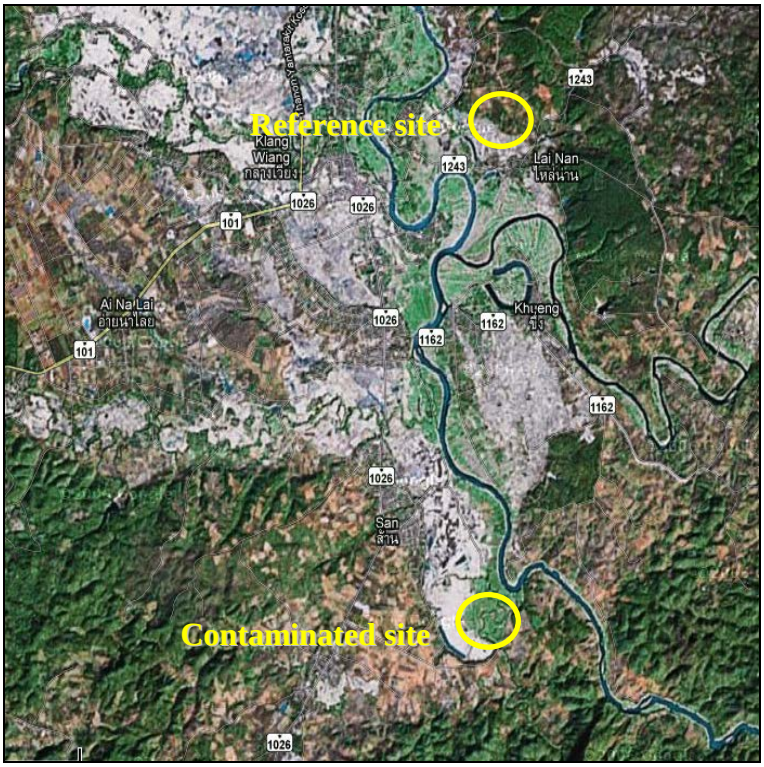
1.1 Contaminated Site

The contaminated site is a paddy field with intensive herbicide usage in Lai-nan Sub-District (Chairaja, interview, October 6, 2009) (location: 47Q 068772, UTM 2054283).

1.2 Reference Site

The reference site is a paddy field with no history of herbicide utilization in San Sub-District (Kamsrikaew, interview, October 6, 2009) (location: 47Q 0686779, UTM 2047187).

These two study sites shared similarity in geography and landscape (Figure 3.1). Therefore, the agricultural practice of using herbicide is the major difference between these sites.



Contaminated Site

location: 47Q 068772, UTM 2054283

Reference Site

location: 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 usage and a reference site which is a paddy field in Lai-nan Sub District with no history of herbicide utilization.

2. Sampling Periods

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

1. July 2010-September 2010 (Late wet period)
2. October 2010-December 2010 (Early dry period)
3. January 2011- March 2011 (Late dry period)
4. April 2011-June 2011 (Early wet period)

Wet and dry seasons in this study were determined based on the climate diagram plot between mean temperature and total rainfall (Walter, Harnickell, and Mueller-Dombois, 1975). The climate diagram indicated that the wet season in this area extended from July to September in 2010 and March to June in 2011, while the dry season extended from October 2010 to February in 2011 (Figure 3.2).

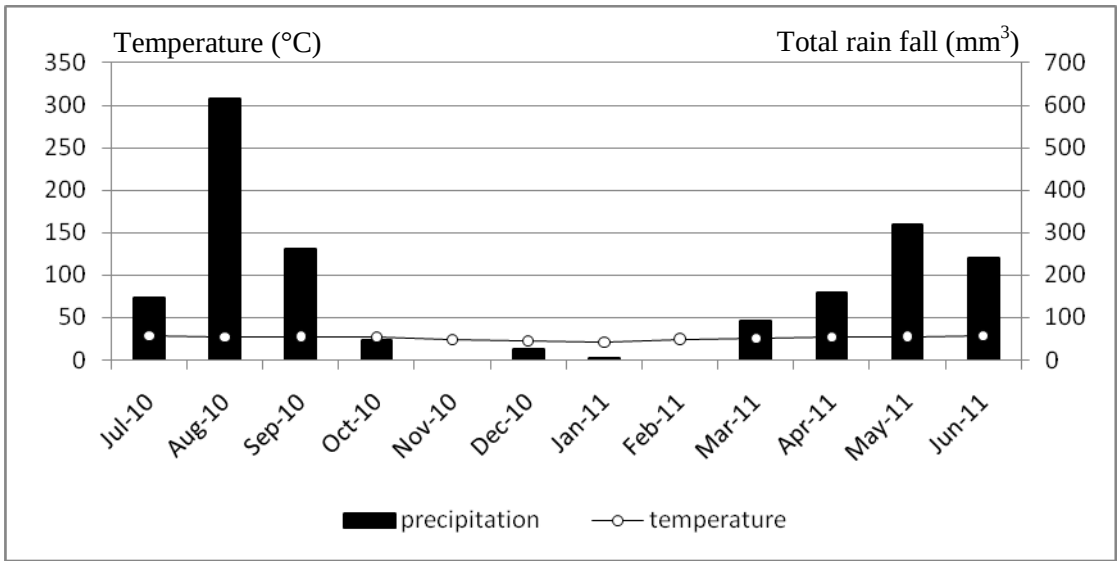


Figure 3.2 Climograph of paddy fields in Nan Province during sampling period (July 2010 – June 2011)

3. Environmental Sample Collection

The composited environmental samples including soil (1 kg) and water (2 L) were field-collected in 4 periods including July 2010, October 2010, January 2011 and April 2011 from both study sites.

After collection, environmental samples were kept in acetone-rinsed high density polyethylene plastic containers, covered with aluminum foil and kept rapidly at -20 °C until further analysis.

Figure 3.3 The composited environmental samples including soil and water were kept from paddy fields in Nan Province.

4. Frog Sample Collection

The rice frogs *Fejervarya limnocharis* were field-collected 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. The frogs were collected by hand at night during visual encounter survey (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% MS-222 solution. The body weight and body length (snout-vent length) were measured. For frog tissue contaminant analysis, 3 male and 3 female frogs per site per period were kept rapidly at -20 °C until further analysis.

5. Herbicide Residue Analysis in Environmental Sample

Herbicide residues in soil and water were analyzed by chromatographic techniques by Central Laboratory (Thailand) Co., Ltd., an ISO/IEC 17025 accredited institutes for food testing by the National Bureau of Laboratory Quality Standards. The overview of chromatographic methods for these herbicides is listed as follows.

5.1 Atrazine

A 500 mL of water sample was pre-treated with sodium chloride (NaCl) and subjected to extraction with dichloromethane (CH_2Cl_2). After drying up the extracted sample in an evaporator, the sample was adjusted to the volume by 2.5 mL ethyl acetate before further analysis. A 10 g of wet soil sample treated with NaCl was extracted with 10 mL acetronitrile (HPLC Grade) before addition of anhydrous magnesium sulfate ($MgSO_4$). The extracted sample was centrifuge at 3,000 rpm (Heraeus®, Megafuge® 1.0 R), 5°C for 5 minutes, and the supernatant was transferred to evaporator under stream of nitrogen gas. Next, the sample was adjusted to volume by ethyl acetate and subjected to treatment with anhydrous $MgSO_4$ and primary secondary amine (PSA) cartridge. The sample was filtered through 0.22 μm syringe filter before further analysis. Residue of atrazine in the extracted sample was quantified by gas chromatography-mass spectrometry (GC-MS; Agilent Technologies 6890 N) using Mass Selective Detector (MSD) and a DB-5ms capillary column (0.25 mm internal diameter x 30 m length with 0.25 μm film thickness) with injection volume of 2 μL and initial flow rate of 1.1 mL/min. The limit of detection (LOD) for atrazine residue was 0.01 mg/L in water and 0.01 mg/kg in soil.

5.2 Glyphosate

A 200 mL of water sample was adjusted to pH 2 and subjected to ion-exchange and clean-up in 8 mL of Chelex 100 column followed by AG1-X8 column. Then, the sample was evaporated to dryness under vacuum and sequentially treated with 5 mL of water and 1 mL of water-methanol-HCl between each evaporation cycle. After the last evaporation under stream of nitrogen, sample was derivatized for 1 hour at 100 °C after treatment with 800 μL of trifluoacetic anhydride and 400 μL of trifluoroethanol. Finally, the sample was evaporated under nitrogen and redissolved in 1 mL of ethyl acetate prior to analysis. A 10 g of soil sample was extracted twice

with 25 mL of 1M NaOH, centrifuged for 10 min at 5,000 rpm and the supernatant was filtered through an F1 Whatman filter. The pooled extracts were treated with 4.2 mL concentrated HCl, diluted with water to the volume of 200 mL and adjusted to pH 2. After 1 hour of incubation at room temperature, a 50 mL of clear upper part of extracts was subjected to similar treatments to water sample. Residue of glyphosate in the extracted sample was determined by high performance liquids chromatography (Agilent HPLC 1100 series) with post-column derivatizer (Pickering PCX 5200) and silica hydrophilic interaction chromatography column (silica HILIC; Atlantis; 2.1 mm internal diameter x 150 mm length and 3 µm film thickness). A 20 µL of sample was injected into the HPLC with post-column derivatizer with 0.2 mL/minute of column flow rate control. The stop time was 12.0 minutes and post time was 3.0 minutes. The column temperature was held at 40 ◦C. The limit of detection (LOD) for glyphosate residue was 0.005 mg/L in water and 0.01 mg/kg in soil samples.

5.3 Paraquat

A 100 mL of water sample was adjusted to pH 9 by 10 N of NaOH , cleaned up through solid phase extraction (SPE) silica and then stored in a mixture of hydrochloric acid and methanol (9:1). After that, the sample was evaporated to dryness and adjusted to volume with 2 mL of mixture of 100 mM ammonium formate pH 3.7 and acetronitrile (3:2). The sample was filtered through syringe nylon filter (0.22 µm) prior to analysis. A 20 g of soil sample was treated with 10 mL of deionized water, 1 mL of octane-2, 35 mL of sulfuric acid and 10 glass beads before refluxed in a close system for 5 hours. After cooled down in room temperature, the sample was filtered through filter paper (Bluchner; NO.4) and Celite before cleaned up through solid phase extraction (SPE) silica. The sample was evaporated to dryness (EyeLa; NVe-2000) and adjusted the volume to 2 mL with mixture of 100 mM ammonium formate pH 3.7 and acetronitrile (3:2). The sample was filtered through syringe nylon filter (0.22 µm) before further analysis. Residue of paraquat was quantified by high performance liquids chromatography with diode array detector (HPLC-DAD; Agilent 1100) and an Atlantis HILIC silica column (2.1 internal diameter x 150 mm length with 3 µm film thickness) with injection volume of 20 µL, flow rate of 0.2 mL/min, stop time 12.00 minutes, post time 3.00 minutes and

temperature column 40.0°C. The spectrum ranged from 190 nm to 400 nm. The limit of detection (LOD) for paraquat was 0.01 mg/L for water and 0.05 mg/kg for soil.

6. Herbicide Residue Analysis in Frog Tissue

The frog samples kept in -20°C were freeze-dried (FreeZone®Model 7753501) for 48 hours to complete dryness and homogenized with the blender (NESCO®). After that, the dry powder-like frog tissue was kept in plastic containers with silica gel, covered with aluminum foil and kept at room temperature (25-35 °C) until further analysis.

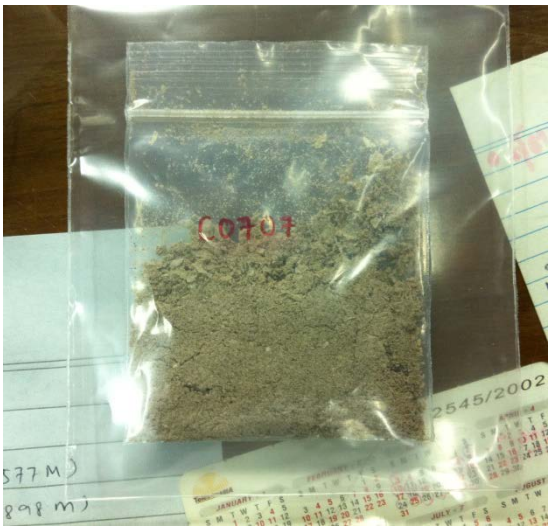


Figure 3.4 Tissue of the rice frog that had been freeze-dried and homogenized

6.1 Atrazine

Determination of atrazine residue was performed by an enzyme-linked immunosorbent assay (ELISA) using a microtiter plate kit of Abraxis LCC (Product No. 520005). The performance data of Abraxis atrazine ELISA kit are as follows. The cross-reactivities with pesticides classes other than triazines have not been observed. The parallel determinations using HPLC and GC/MS methods showed a good correlation in the atrazine concentration. This Abraxis atrazine ELISA kit is provided for dertermination of atrazine in water sample. Therefore the sample of frog tissue was extracted and reconstituted in water before assay by this kit. The extraction protocol was modified from Jacomini et al. (2003) as follows. A 100 mg of frog tissue was mixed with 1 mL of ultrapure H_2O (HPLC Grade; Merck). After that,

200 µL of 1.5 M NaOH and 4 mL of dichloromethane (HPLC Grade; Fisher®) were added into the sample followed by shaking vigorously (Vortex Genie 2) for 20 minutes. The sample was centrifuged (WiseSpin®CF10) at 1,800 xg for 5 minutes at room temperature (25° C). Then, 3 mL of organic phase was transfered into a new clean tube and evaporated under stream of N_2 (Turbo Vap® II) until dry. The sample was reconstituted with 100 µL of methanol and shaked vigorously. Afterward, 900 µL of dH_2O was added and mixed thoroughly. Finally, the sample with final volume of 1 mL was stored at -20 ^oC until further analysis. To check for recovery of extraction, 5 ng of standard atrazine (50 µL of 100 ng/mL atrazine solution) was added to a representative dry sample before proceeding with the subsequent steps.

For enzyme-linked immunosorbent assay procedure, the 96-well microtiter plate coated with rabbit anti-triazine antibodies and all reagents were adjusted to room temperature (25 ^oC) before each use. Initially, 25 µL of assay buffer was added into each well, followed by duplicated addition of 25 µL of standard solutions (atrazine standard 0- 6; including S_0= 0 ng/mL, S_1= 0.05 ng/mL, S_2= 0.1 ng/mL, S_3= 0.25 ng/mL, S_4= 1.0 ng/mL, S_5= 2.5 ng/mL and S_6= 5 ng/mL) or the extracted-samples. Then, 50 µL of triazine-horseradish peroxidase-conjugate was added into each individual well to compete with atrazine residue in standards/samples for binding with antibodies. The plate was incubated on an orbital shaker (Mini-Rock shaker PSU-2T BIOSAN) at room temperature (25 ^oC) for 30 minutes. After incubation, the plate content was discarded and the plate was washed 3 times with washing buffer solution. Then, 100 µL of substrate/color solution (hydrogen peroxide and a chromogen: 3,3',5,5'- tetramethylbenzidine; TMB) was added into each individual well, and the plate was incubated on the orbital shaker at room temperature (25 ^oC) for 15 minutes. After incubation, 50 µL of stop solution (diluted sulfuric acid) was added into the wells. Finally, the microtiter plate was subjected to absorbance measurement at 450 nm by a microplate reader (Multiskan EX).

To calculate for atrazine residue, means absorbance of the duplicated standards/samples were calculated. Next, % B/B_0 was calculate by dividing the mean absorbance of each standards/samples with the mean value of standard 0 (0 ng/mL). The standard curve was constructed by plotting the % B/B_0 for each standard (standard 0-6) on Y axis (vertical linear scale) and the corresponding atrazine

concentration on X axis (horizontal logarithmic scale) on the graph. The standard calibration curve of atrazine was linear with the r^2 of 0.97035 to 0.99660. Atrazine of each sample could be interpolated based on an equation derived from the standard curve: **Y = a ln(X) + b**, when Y is $\%B/B_0$ of the sample and X is the corresponding atrazine concentration in ppb (ng/mL). After the extraction factor was taken into consideration, the atrazine concentration in frog tissue was present in the unit of µg/kg dry weight. The detection limit (90% B/B_0) for atrazine was calculated to be 0.53 µg/kg dry weight for frog tissue sample. The recovery of extraction calculated from a proportion of atrazine detected in the atrazine-spike sample and the actual amount of spiked atrazine (5 ng) was 54.75 %.

6.2 Glyphosate

Residue of glyphosate was determined by a glyphosate ELISA kit of Abraxis LCC (Product No. 500086). This Abraxis glyphosate ELISA kit is provided for determination of glyphosate in water sample. Therefore the sample of frog tissue was extracted and reconstituted with water before assay with this kit. The extraction protocol was modified from Alferness and Iwata (1994) as follows. A 100 mg of frog tissue was mixed with 200 µL of ultrapure H_2O (HPLC Grade; Merck) and shaken vigorously for 1 min. After that, 100 µL of chloroform (Merck) and 500 µL of 0.1 N HCl were added and the sample was shaken vigorously for 5 min. Next, the 0.004 g of sodium sulfate (Merck) was added and the sample was shaken vigorously for another 2 min. Then, the sample was centrifuged at 1,000 xg for 10 min at room temperature (25 ^oC), and 400 µL of the aqueous extraction was transferred into a clean tube. The sample was re-extracted by adding 500 µL of chloroform and shaking vigorously for 2 min. Afterward the re-extracted sample was centrifuged at 1,000 xg for 10 min at room temperature (25 ^oC), and 350 µL of the aqueous extraction was pooled with the first aqueous extraction to get the total volume of 750 µL. The sample pH was adjusted by 1N NaOH to 6-8. Finally, the sample was stored at -20 ^oC until further analysis. To check for recovery of extraction, 0.8 ng of standard glyphosate (200 µL of 4 ng/mL glyphosate solution) was add to a representative sample instead of 200 µL dH_2O before proceeding with the subsequent steps.

To proceed with ELISA procedure, glyphosate standards and the extracted samples were subjected to derivatization as follows. A 250 mL of glyphosate standard solutions (standard 0-5; including S_0= 0 ng/mL, S_1= 0.75 ng/mL, S_2= 0.2 ng/mL, S_3= 0.5 ng/mL, S_4= 1.0 ng/mL and S_5= 4 ng/mL) and the extracted samples were loaded in to a glass tube and mixed with 1 mL of assay buffer. Then, 100 µL of derivatization reagent was added into each tube, and the tube was shaken vigorously for 15 seconds. After that, the derivertized standards/samples were kept at room temperature (25 ^{o}C) for 10 minutes. A 50 mL of rabbit anti-glyphosate antibody in buffered saline solution was added into each well of the 96-well microtiter plate coated with goat anti-rabbit. Next, A 50 µL of the derivatized standards/samples was added in duplication into the well, and the plate was incubated on a orbital shaker (Mini-Rock shaker PSU-2T BIOSAN) at room temperature for 30 minutes. After incubation, 50 uL of the glyphosate enzyme conjugate (horseradish peroxidase; HRP labeled glyphosate analog dilute in a buffered solution) was added into each individual well, and the plate was incubated on the orbital shaer at room temperature for 97 minutes. After incubation, the plate was washed 3 times with washing buffer. Then, 150 µL of substrate/color solution (hydrogen peroxide and a chromogen: 3,3',5,5'-tetramethylbenzidine; TMB) was added into each well, and the plate was incubated on the orbital shaker at room temperature for 20 minutes. After incubation, 100 µL of stop solution (diluted sulfuric acid) was added into each well, and the plate content was measured for an absorbance at 450 nm using a microplate reader.

To calculate glyphosate concentration, mean absorbance of the duplicated standards/samples were calculated as first. Then, % B/B_0 was calculated by dividing the mean absorbance of each standards/samples with the mean absorbance of standard 0 (0 ng/mL). Afterward, a standard curve was made by plotting the % B/B_0 for each standard (standard 0-5) on Y axis and the corresponding logarithmic glyphosate concentration on X axis. The standard calibration curve of glyphosate was linear with r^2 of 0.99059 to 0.99498. Glyphosate concentration in each sample could be interpolated based on an equation derived from the standard curve: **$Y = a \ln(X) + b$;** when Y is $\%B/B_0$ of the sample and X is the corresponding glyphosate concentration in ppb (ng/mL). After the extraction factor was taken into consideration, the glyphosate concentration in frog tissue was present in the unit of µg/kg dry weight.

The detection limit (90% B/B_0) for glyphosate was was calculated to be 0.45 µg/kg dry weight of frog tissue sample. The recovery of extraction of 131.76% was calculated from percentage of the detected amount of glyphosate in a sample spiked with standard glyphosate compared to the original amount of glyphosate added to this sample (0.8 ng).

6.3 Paraquat

Determination of paraquat in frog tissue extract was carried out using ELISA microtiter plate kit of Abnova (Catalog Number KA1424). This paraquat ELISA kit is designed for dertermination of paraquat in water sample. Therefore the sample of frog tissue was extracted and reconstituted with water in advance. The extraction protocol was modified from Brown et al. (1996) and Quick et al. (1990) as follows. One hundred milligrams of frog tissue powder was mixed with 200 µL of ultrapure H_2O (HPLC Grade; Merck) in a microtube and then shaken vigorously for 1 min. Next, 200 µL of hexane (Merck) and 600 µL of 10 % trichloroacetic acid (TCA; Merck) were added and then shaken vigorously for 5 min. The sample was centrifuged at 2,000 xg for 15 min at room temperature (25 ^oC). Afterward, 550 µL of aqueous extraction was transfered into a new clean microtube. The residue sample was extracted once more by adding 250 µL of 10% trichloroacetic acid (TCA) and shaking vigorously for 5 min. Next, the sample was centrifuged at 2,000 xg for 15 min at room temperature. After that, 200 µL of aqueous extract was tranfered and combined with the first extract in order to get the total volume of 750 µL). This aqueous phase was extracted to remove fat by adding 400 µL of hexane and shaking vigorously for 5 min. After that, the sample was centrifuged at 2,000 xg for 15 min at room temperature, and 700 µL of aqueous extract was tranferred into a new clean microtube. Three hundred microliters of 2M Tris-hydrochloric acid buffer (pH 8.5) was added into this extract and the sample was adjusted to pH 7 with 6 N hydrochroric acid (HCl). Finally, the sample was stored at -20 ^oC until further analysis. To check for recovery of extraction, 1.5 ng of standard paraquat (200 µL of 7.5 ng/mL paraquat solution) was added to a representative sample instead of 200 µL of dH_2O before proceeding with the subsequent steps.

For ELISA procedure, 25 µL of standard solutions (paraquat standard 0-4; including S_0= 0 ng/mL, S_1= 0.375 ng/mL, S_2= 0.75 ng/mL, S_3= 2.5 ng/mL and S_4= 7.5 ng/mL) and the extracted samples were added in duplication into each well of the 96-well microtiter plate coated with rabbit anti-paraquat antibodies. Next, 100 µL of paraquat-horseradish peroxidase conjugate (PRQ-HRP) was added into each individual well, and the plate was incubated on an orbital shaker (Mini-Rock shaker PSU-2T BIOSAN) at room temperature for 30 minutes. After incubation, the plate was washed 3 times with washing buffer to remove the unbound materials. A 100 mL of substrate/color solution (hydrogen peroxide and a chromogen: 3,3',5,5'-tetramethylbenzidine; TMB) was added into each individual well, and the plate was incubated on the orbital shaker at room temperature for 15 minutes. After incubation, 100 µL of stop solution (3M HCl) was added into each well, and the well content was measured for an absorbance at 450 nm using a microplate ELISA reader.

To calculate concentration of paraquat, mean absorbance of the duplicated standards/samples were calculated first. Afterward, % inhibition was calculated as follows.

% inhibition = 100 – (mean absorbance of sample / mean absorbance of S_0) X 100

A standard curve was constructed by plotting the % inhibition of each standard (standard 0-4) on Y axis and the corresponding paraquat concentration on X axis. The standard calibration curve of glyphosate was linear with r^2 of 0.97754 to 0.99938. Paraquat concentration in each sample could be interpolated based on equation derived from the standard curve: **Y = a ln(X) + b;** when Y is the % inhibition of the sample and X is the corresponding paraquat concentration in ppb (ng/mL). After the extraction factor was taken into consideration, the paraquat concentration in frog tissue was shown in a unit of µg/kg dry weight. The detection limit for paraquat was calculated to be 0.61 µg/kg dry weight of frog tissue sample. The recovery of extraction of 94.91 % was calculated from percentage of the detected amount of paraquat in a sample spiked with standard paraquat compared to the original amount of paraquat added to this sample (0.15 ng).

7. Statistical Analyses

All parameters were tested for normal distribution and homogeneity of variance before proceeding with the required analysis. Levels of herbicide residue in the rice frog were compared between male and female frogs in each site by *t*-test or Mann-Whitney rank sum test. In case of no significant sex related difference, the male and female data were pooled. The data of herbicide residues in each period were compare between sites by *t*-test or Mann-Whitney rank sum test. The levels of herbicide contamination in each site were compared among periods by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on rank. In case of no significant seasonal difference, the data from every periods was pooled and compared between sites by *t*-test or Mann-Whitney rank sum test. The Sigma Plot 11.0 was used as a statistical software for these tests.

8. The Biological Concentration Factor

The biological concentration factor (BCF) was determined by dividing the herbicide levels found in biological sample by the levels found in the environmental sample (or herbicide residue in frog tissue / herbicide residue in environment).

Results

1. Environmental Contamination (Water and Soil Samples)

The results of environmental contaminant analysis showed that only atrazine residue was present in detectable amount during these study periods (Table 3.1), while levels of glyphosate and paraquat were below limit of detection in every samples and periods (Table 3.2 and Table 3.3).

Table 3.1 Atrazine contamination in environmental samples from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/Year	Reference site		Contaminated site	
		Water (mg/L) N=1	Soil (mg/kg) N=1	Water (mg/L) N=1	Soil (mg/kg) N=1
Late wet	July 2010	<0.01	<0.01	<0.01	<0.01
Early dry	October 2010	<0.01	<0.01	<0.01	<0.01
Late dry	January 2011	N/A	<0.01	**0.15**	<0.01
Early wet	April 2011	<0.01	<0.01	<0.01	<0.01

Remarks:

- Analyzed by an in-house method of Central Laboratory (Thailand) Co., Ltd., based on gas chromatography mass spectrometry (GC-MS Agilent Technologies 6890N)
- N/A = Data not available
- Limit of detection (LOD): water = 0.01 mg/L, soil = 0.01 mg/kg,

Table 3.2 Glyphosate contamination in environmental samples from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/Year	Reference site		Contaminated site	
		Water (mg/L) N=1	Soil (mg/kg) N=1	Water (mg/L) N=1	Soil (mg/kg) N=1
Late wet	July 2010	<0.005	<0.01	<0.005	<0.01
Early dry	October 2010	<0.005	<0.01	<0.005	<0.01
Late dry	January 2011	N/A	<0.01	<0.005	<0.01
Early wet	April 2011	<0.005	<0.01	<0.005	<0.01

Remarks:

- Analyzed by an in-house method of Central Laboratory (Thailand) Co., Ltd., based on high performance liquids chromatography (HPLC 1100 series / Pickering Laboratories PCS 5200 Post column derivatizer)
- N/A = Data not available
- Limit of detection (LOD): water = 0.005 mg/L, soil = 0.01 mg/kg

Table 3.3 Paraquat contamination in environmental samples from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/Year	Reference site		Contaminated site	
		Water (mg/L) N=1	Soil (mg/kg) N=1	Water (mg/L) N=1	Soil (mg/kg) N=1
Late wet	July 2010	<0.01	<0.05	<0.01	<0.05
Early dry	October 2010	<0.01	<0.05	<0.01	<0.05
Late dry	January 2011	N/A	<0.05	<0.01	<0.05
Early wet	April 2011	<0.01	<0.05	<0.01	<0.05

Remarks:

- Analyzed by In-house method of Central Laboratory (Thailand) Co., Ltd., based on high performance liquids chromatography for (HPLC-DAD ; Agilent 1100)
- N/A = Data not available
- Limit of detection (LOD): soil = 0.05 mg/kg, water = 0.01 mg/L

2. Frog Tissue Contamination

2.1 Atrazine

The results of frog tissue contaminant analysis showed that atrazine was found in tissue of the rice frog in both contaminated site and reference site (Table 3.4). There was no significant difference between atrazine concentration in male and female frog (Mann-Whitney rank sum test, $p>0.05$). Therefore the data between male and female was pooled. In the contaminated site, level of atrazine ranged from 4.12 ± 1.61 µg/kg in October 2010 to 14.10 ± 5.83 µg/kg in July 2010. In the reference site, the levels of atrazine ranged from 3.62 ± 0.50 µg/kg in April 2011 to 7.26 ± 1.96 µg/kg in October 2010. The level of atrazine contamination in tissue of frogs from the contaminated site was not significantly different from those of the reference site (8.70 ± 1.44 µg/kg in the contaminated site vs. 5.05 ± 0.67 µg/kg in the reference site; Mann-Whitney Rank Sum Test, $p>0.05$) (Table 3.4).

Table 3.4 Atrazine residues (Mean±SEM) in tissues of the rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/Year	Reference site (µg/kg)	Contaminated site (µg/kg)
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)
overall	July 2010-June 2011	5.05 ± 0.67 (N=24)	8.70 ± 1.44 (N=24)

Remarks:

- There was no significant difference between sites in each sampling period.
- Site related difference was analyzed by *t*-test or Mann-Whitney rank sum test.
- Seasonal difference in each site was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on rank test.

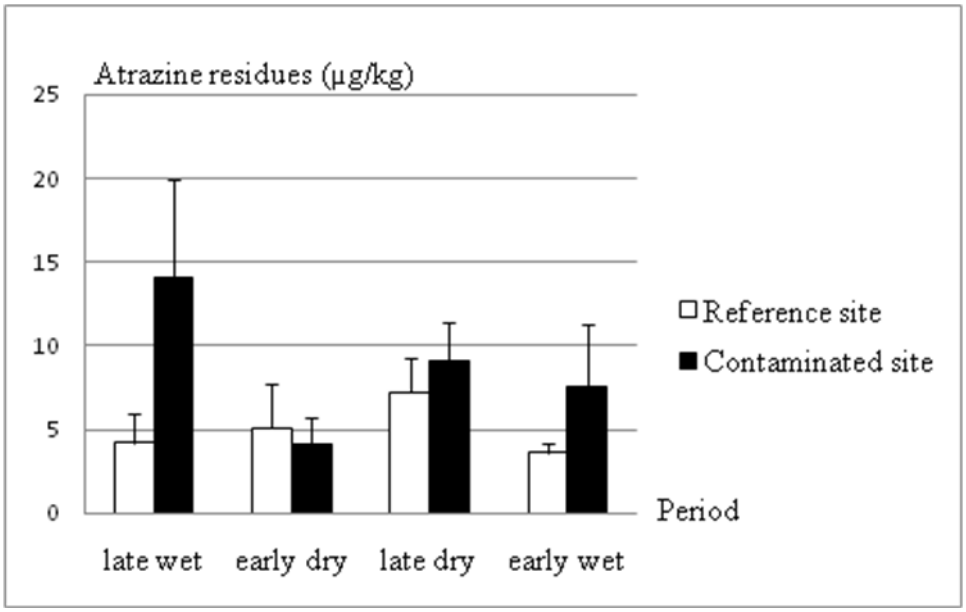


Figure 3.5 Atrazine residues (Mean±SEM) in tissues of the rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remarks:

- There was no significant difference between sites in each sampling period.
- Site related difference was analyzed by *t*-test or Mann-Whitney rank sum test.
- Seasonal difference in each site was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on rank test.

2.2 Glyphosate

The results of frog tissue contaminant analysis showed that detectable amount of glyphosate was found in tissue of the rice frog at both contaminated and reference sites (Table 3.5). There was no significant difference between glyphosate concentration in male and female frogs (Mann-Whitney rank sum test, $p>0.05$). Therefore, the data of both sexes was pooled. In the contaminated site frogs, level of glyphosate ranged from 5.48 ± 2.74 µg/kg in July 2010 to 10.90 ± 6.29 µg/kg in October 2010. In the reference site frogs, level of glyphosate ranged from 4.91 ± 1.17 µg/kg in July 2010 to 8.26 ± 2.64 µg/kg in October 2010. The level of glyphosate residue in frogs from the contaminated site was not significantly different from those of the reference site (7.90 ± 1.68 µg/kg in contaminated site vs. 6.19 ± 1.31 µg/kg in reference site; Mann-Whitney rank sum test, $p>0.05$; Table 3.5).

Table 3.5 Glyphosate residues (Mean±SEM) in tissues of the rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/Year	Reference site (µg/kg)	Contaminated site (µg/kg)
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)
overall	July 2010-June 2011	6.19 ± 1.31 (N=22)	7.90 ± 1.68 (N=22)

Remarks:

- There was no significant difference between sites in each sampling period.
- Site related difference was analyzed by *t*-test or Mann-Whitney rank sum test.
- Seasonal difference in each site was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on rank test.

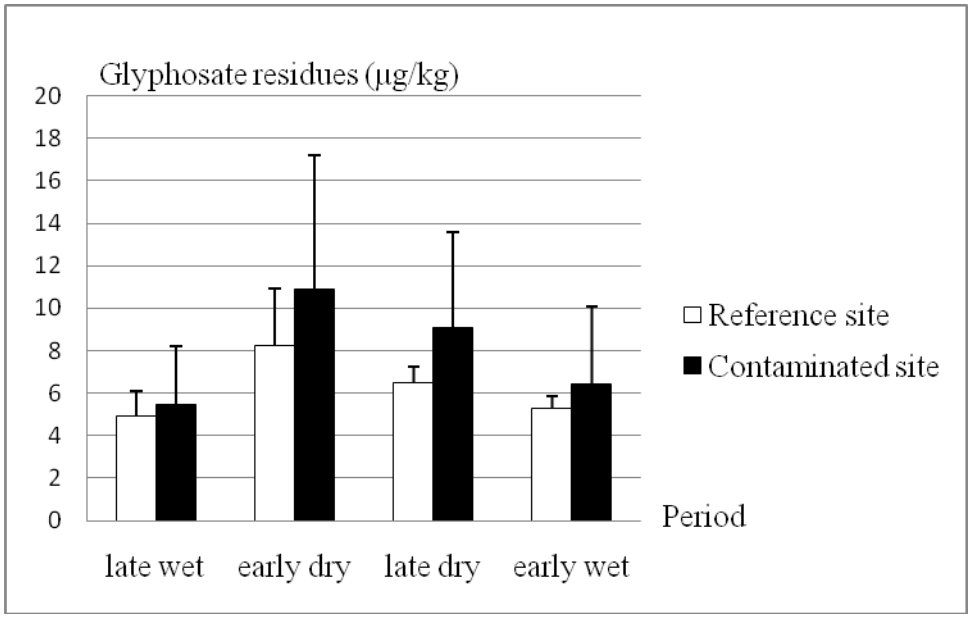


Figure 3.6 Glyphosate residues (Mean±SEM) in tissues of the rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand.

Remarks:

- There was no significant difference between sites in each sampling period.
- Site related difference was analyzed by *t*-test or Mann-Whitney rank sum test.
- Seasonal difference in each site was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on rank test.

2.3 Paraquat

The results of herbicide residue analysis in frog tissue showed that paraquat could be found in tissue of the rice frog at both contaminated and reference sites (Table 3.6). There was significant difference between paraquat concentration in male and female frogs (Mann-Whitney rank sum test, $p<0.05$). Therefore, the data of male and female frogs were analyzed seperately.

In male frogs, level of paraquat ranged from 38.83 ± 4.26 µg/kg in January 2011 to 115.16 ± 50.74 µg/kg in October 2010 at the contaminated site, and 48.63 ± 5.11 µg/kg in July 2010 to 86.68 ± 33.26 µg/kg in October 2010 at the reference site. Level of paraquat contamination in male frog caught in July 2010 was significantly higher in the contaminated site than those in the reference site (83.32 ± 16.49 µg/kg vs. 56.67 ± 4.50 µg/kg; *t*-test, $p<0.05$). However, overall mean of paraquat residue in male frog was not significantly different between the contaminated site and the reference site (88.80 ± 13.07 µg/kg in the contaminated site vs. 57.75 ± 7.04 µg/kg in the reference site; Mann-Whitney rank sum test, $p>0.05$; Table 3.6).

Table 3.6 Paraquat residues (Mean±SEM) in tissues of the male rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/Year	Reference site (µg/kg)	Contaminated site (µg/kg)
Late wet	July 2010	48.63 ± 5.11 (N=3)	84.82 ± 7.59* (N=3)
Early dry	October 2010	86.68 ± 33.26 (N=2)	115.16 ± 50.74 (N=3)
Late dry	January 2011	51.24 ± 4.54 (N=3)	38.83 ± 4.26 (N=2)
Early wet	April 2011	54.11 ± 12.05 (N=3)	79.64 ± 34.26 (N=3)
overall	July 2010-June 2011	57.75 ± 7.04 (N=11)	83.32 ± 16.49 (N=11)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site from the reference site.
- Site related difference was analyzed by *t*-test or Mann-Whitney rank sum test.
- Seasonal difference in each site was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on rank test.

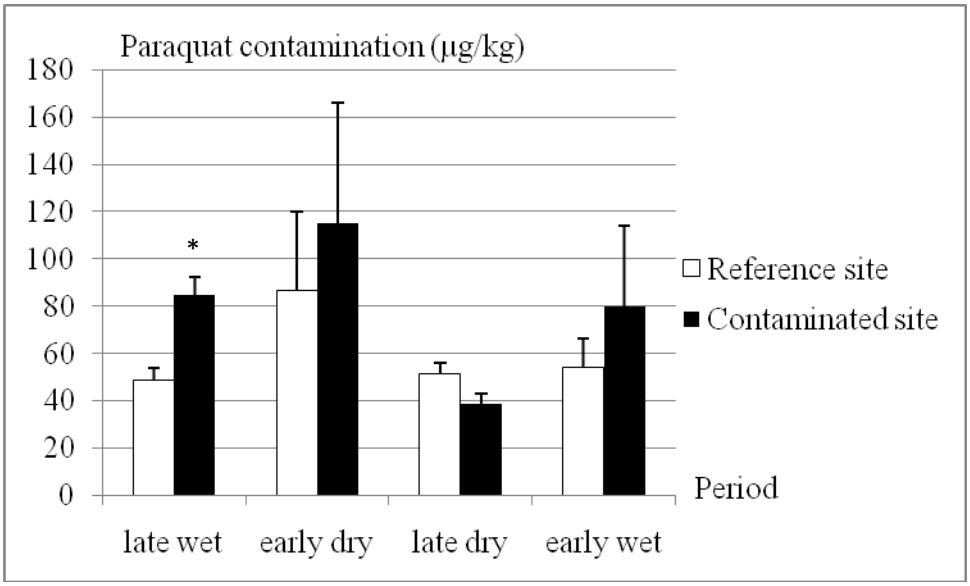


Figure 3.7 Paraquat residues (Mean±SEM) in tissues of the male rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site from the reference site.
- Site related difference was analyzed by *t*-test or Mann-Whitney rank sum test.
- Seasonal difference in each site was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on rank test.

In female frogs, level of paraquat ranged from 44.15 ± 8.27 µg/kg in July 2010 to 166.07 ± 46.27 µg/kg in January 2011 at the contaminated site, and 38.51 ± 4.06 µg/kg in April 2011 to 70.93 ± 6.90 µg/kg in July 2010 at the reference site. There was no significant difference in overall mean of paraquat contamination in female frog between the contaminated site and the reference site (94.84 ± 21.39 µg/kg in the contaminated site vs. 55.58 ± 5.93 µg/kg in the reference site; Mann-Whitney rank sum test, $p>0.05$; Table 3.7).

Table 3.7 Paraquat residues (Mean±SEM) in tissues of the female rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/Year	Reference site (µg/kg)	Contaminated site (µg/kg)
Late wet	July 2010	70.93 ± 6.90 (N=3)	44.15 ± 8.27 (N=3)
Early dry	October 2010	58.99 ± 16.08 (N=3)	111.02 ± 1.48 (N=2)
Late dry	January 2011	48.21 ± 9.27 (N=3)	166.07 ± 46.27 (N=3)
Early wet	April 2011	38.51 ± 4.06 (N=2)	47.84 ± 5.33 (N=2)
overall	July 2010-June 2011	55.58 ± 5.93 (N=11)	94.84 ± 21.39 (N=10)

Remarks:

- There was no significant difference between sites in each sampling period.
- Site related difference was analyzed by *t*-test or Mann-Whitney rank sum test.
- Seasonal difference in each site was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on rank test.

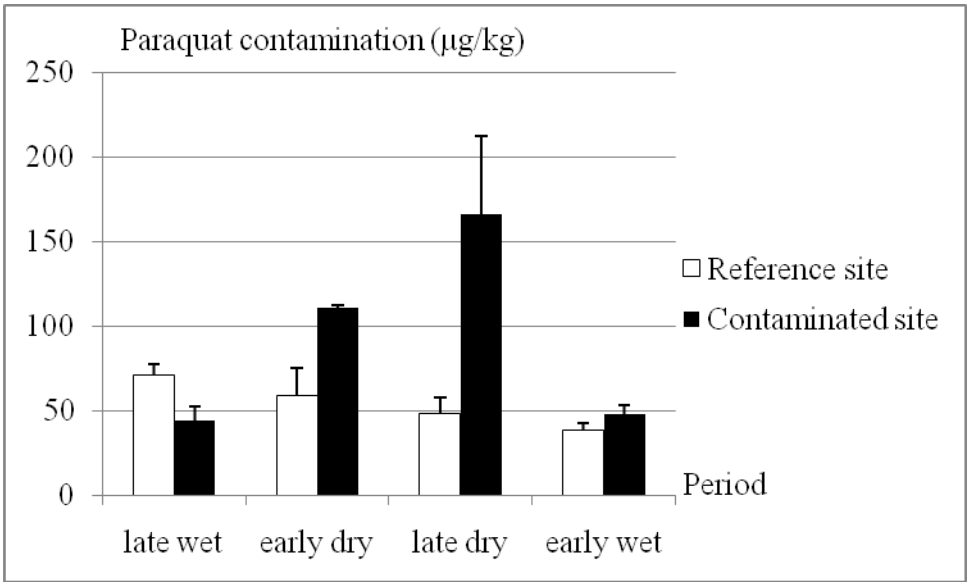


Figure 3.8 Paraquat residues in tissues of the female rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand.

Remarks:

- There was no significant difference between sites in each sampling period.
- Site related difference was analyzed by *t*-test or Mann-Whitney rank sum test.
- Seasonal difference in each site was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on rank test.

3. The Biological Concentration Factor

The biological concentration factor (BCF) of atrazine contamination was 0.058 in the contaminated site. However, the BCF of atrazine in reference site, BCF of glyphosate and BCF of paraquat could not be determined since no data on environmental contamination level was available.

Discussion

During the field surveys, evidence of herbicide (atrazine, glyphosate and paraquat) utilization in contaminated site was observed by researcher. However, results of contaminant analysis showed that only atrazine residue was present in the detectable amount during these study periods (Table 3.1), while levels of glyphosate and paraquat were below the limit of detection in every samples and periods. The atrazine concentration found in this study (0.15 mg/L) was much higher than the Maximum Contaminant Level (MCL) of US EPA in drinking water (0.003 mg/L or 3 ppb; US EPA, 2009) and also higher than the ecological risk concentration of 10-20 µg/L (US EPA, 2003). This result raises a concern over the potential effect of atrazine on non-target organisms including amphibians.

The non-detectable amount of atrazine seen in this study is similar to a study by Wehtje et al. (1981) in which herbicide residues were not detected in ground water of the Platte Valley, Nebraska eventhough the utilization was evidenced. It was believed that the dose use was relatively low so that the herbicides were diluted with water and present in concentration below the limit of detection.

In this study, it is possible that herbicides used in this area have a relatively short half-life and their residues were degraded by photolysis or metabolic reactions in soil and water (Scribner et al., 2000; Wehtje et al., 1981). In addition, technical difficulties of chromatographic techniques can play roles in limit of detection since several studies had reported on the complicated steps and high limit of detection in herbicide residue analysis in environment (Balinova, 1996).

It is of interest to note that detectable amount of atrazine can be found in water in agricultural areas. Similar to this study, McMahon et al. (2005) reported that atrazine residue can be found in sediment of the river near contaminated site in early rainy season. However, the contaminant levels were relatively lower than this study (10 ng/L in water and 1.1 µg/kg in sediment). Hayes et al. (2003) showed that atrazine could remain in agricultural area and also contaminated to other natural area with the range of contamination between 0.2 to 6.7 µg/L. They also indicated that this atrazine contamination level (≥0.1 µg/L) can effect on gonadal development of the leopard frog in natural habitat.

From this study, the 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. Although the extent of 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).

Interestingly, this study found that all three herbicides including atrazine glyphosate and paraquat were contaminated in frog tissues at both reference and contaminated sites. Unfortunately, only a few previous studies indicated the level of these herbicide residues in frog. However, based on Allran and Karasov (2000) study, tadpole exposed to 20 µg/L of atrazine could accumulate atrazine in its tissue upto 128 µg/L, indicating and supporting the current study that these herbicides could in fact contaminate in the amphibian tissue.

Although average level of atrazine was not significantly different between sites, but the residues in contaminated site tend to be higher than those from reference site. It is possible that the relatively higher level of atrazine in tissue of the rice frog may negatively affect the rice frog health and body functions since atrazine is a known endocrine-disrupting chemicals that can affect on reproductive system of amphibians (Kavlock, 2001; Hayes et al., 2002, 2006, 2010; Kloas and Lutz, 2006. However, the biological concentration factor of only 0.058 indicating that the bioaccumulation of atrazine in the rice frog is quite unlikely. Unlike this study, previous studies reported that bioconcentration factor (BCF) of atrazine in other amphibians were quite substantial. The African clawed frog (*Xenopus laevis*) exposed to 1.9 nmol/L of c-14 atrazine showed the BCF is 1.6 (Edginton and Rouleau, 2005), while the leopard frog (*Rana pipiens*) tadpole exposed to 20 µg/L of atrazine showed the BCF of more than 6 (Allran and Karasov, 2000).

Level of glyphosate was not significantly different between sites, while level of paraquat in frog from the contaminated site tended to be higher than the reference site with the significant difference in male frog caught in late wet period (July 2010). Unfortunately, there was no direct evidence on contamination of these herbicides in frog tissue in previous researches. However, in other aquatic vertebrates, Wang et al.

(1991) reported that, using radioactive labeled glyphosate, fishes exposed to glyphosate at 0.05 mg/L could accumulate upto 0.66 µg/L in carp and 1.30 µg/L in tilapia after 7 days of exposure. These results show that the glyphosate can actually accumulate in tissues of vertebrates. In case of paraquat, previous study using autoradiography of ^{14}c paraquat revealed that paraquat could accumulate in melanin tissue of frog *Rana temporaria* (Lindquist et al., 1988). These evidences of the potential accumulation of glyphosate and paraquat supported the current study that glyphosate and paraquat could contaminate in nontarget organism tissue.

The result of contaminant analysis in frog tissue also showed trend of seasonal difference in herbicide contamination. This may be due to the different agricultural acitivities between these periods. High level of atrazine was found during late wet period (July-September) when an onset of agricultural acitivities with more intensive use of herbicide was evidenced. High levels of glyphosate and paraquat in dry season (October-March) was found when an intensive use of herbicides for non annual crops was evidenced. This also corresponded with the field observation that herbicide utilization was relatively rare in early wet period (April-June) when no agricultural activity was found since the land was prepared for the next crop cycle. In general, the current finding indicates that the rice frog is a good biological receptor and/or accumulator that can reflect the pattern of herbicide utilization in this area.

This study also showed that these herbicides (atrazine, glyphosate and paraquat) could be found in tissue of the rice frog from both contaminated site and reference site. The herbicide utilization in the contaminated site was obviously evidenced during the field surveys, whereas no history or no evidence of herbicide usage in the reference site was found. It is possible that these herbicides were introduced into this reference site by other means. In the dry periods, the reference site was irrigated with water from Nan Rivers for agricultural purpose. The concurrent study reported that atrazine residue was found in Nan River, especially during the dry season (Maneein et al., 2011 and Senarat, 2011). Furthermore, physical factors such as precipitations (rain and fog) could play role in transferring herbicide from one area to other areas. Previous study by Goolsby et al., (1997) showed that herbicides (atrazine) and it metabolites could be found in rainfall. 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.

Since the rice frog was used as food for local people, herbicide contamination in frog could directly pose serious threat to human who consume this frog. Fortunately, ranges of atrazine (1.30-26.55 µg/kg dry weight) and glyphosate (1.69-23.12 µg/kg dry weight) residues in frog tissue were below the maximum residue limit in food for both atrazine (40 µg/kg wet weight or equivalent to 73.58 µg/kg dry weight of frog tissue; Health Canada, 2010) and glyphosate (50 µg/kg wet weight or equivalent to 91.98 µg/kg dry weight of frog tissue; Codex Alimetarius, 2006). However, it is of importance to note that the maximum residue limit of paraquat proposed by Codex Alimetarius (2006) was 5 µg/kg wet weight or equivalent to 9.20 µg/kg dry weight of frog tissue. Therefore, paraquat residues found in the rice frog (32.42-2,270.28 µg/kg dry weight) were much higher than the maximum residue limit, especially in late dry period when 2,270.28 µg/kg of paraquat (or 246 times of the maximum residue limit) was found in frog from the contaminated site. Since the boiling point of paraquat is 300 °C (International Program on Chemical Safety, 2012), its degradation by heat after cooking at high temperature is highly unlikely. Therefore, it is crucial to create public awareness that consumption of the rice frogs caught from these agricultural areas should be avoided.

Conclusion

In this part of the study, contaminant analysis of environmental samples showed that atrazine herbicide could be detected in agricultural area environment. Analysis in the tissue of the vertebrate, the results showed that the all three herbicides (atrazine, glyphosate, paraquat) were contaminated in frog tissue with the significant site-related difference in paraquat. These findings indicate that the intensive herbicide utilization in paddy fields in Nan Province can cause environmental contamination of the herbicides and lead to herbicide contamination in frogs or the vertebrates living in those areas. This contamination poses some concerns over the potential contamination in other non-target organisms including human living in those areas.

CHAPTER IV

INFLUENCE OF HERBICIDES ON MORPHOLOGY OF RICE FROG *Fejervarya limnocharis* IN PADDY FIELDS, NAN PROVINCE

Introduction

Currently, environmental contamination has become one of the global environmental problems occurring in all over the world. The sources of contamination include both industrial and agricultural activities. In agriculture, use of agrochemicals, especially pesticides, is regarded as one of the prominent means of 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, their residues are likely to be present in the agricultural areas. Non-target organisms living in these areas are thus susceptible to an exposure with the pesticide residues and an adverse effect from this contamination (Hughes, 1996).

Pesticide contamination may affect on any vertebrates living in vicinity of the agricultural areas including human. Therefore, it is essential to monitor the extent of contamination in physical environment as well as in a representative vertebrate living in the affected area. Also, since non-human vertebrates share several similar structures and function of their organs to those of human, monitoring adverse changes in the animal life could be used as an early warning sign of potential impacts from pesticide contamination to human (NRC, 1991).

Amphibians are regarded as one of a suitable sentinel species for environmental contamination. Their natural habitats include agricultural areas where they can be exposed to pesticides (Khan and Law, 2005). Their existence both in water and on land makes them susceptible to pesticide exposure through several routes, especially through their semi-permeable skin (Duellman and Trueb, 1994; Roy, 2002; Quaranta et al., 2009). Studies on morphology of amphibian can thus be used to evaluate long-term impact of pesticide contamination on vertebrates (Ouellet M, 2000; Du Preez et al., 2005; Soderman et al., 2007). In addition, since amphibian decline has become a global problem for a few decades and the environmental contamination is regarded as 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, climate change, infection, habitat destruction, or synergistic of these factors (Brige et al., 2000).

In Thailand, especially Nan Province in the north, there are a lot of agricultural areas where pesticides have been used for a long time. Most of pesticides used in this area include herbicides namely atrazine, glyphosate and paraquat. Atrazine is a known endocrine-disrupting chemicals that can affect on reproductive system of amphibians (Kavlock, 2001; Hayes et al., 2002, 2006, 2010; Kloas and Lutz, 2006) while glyphosate and paraquat are also found to show similar adverse effect on amphibians (Relyea, 2005; Quassinti et al., 2009). 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. Therefore, it is crucial to examine whether utilization of these herbicides will have any impact on the amphibians living in these areas.

In this study, the rice frog *Fejervarya limnocharis* was used as a sentinel species of environmental health hazards from the herbicide contamination since stable population of *F. limnocharis* can be found in the agriculture areas making it susceptible to long term exposure and accumulation of xenobiotics (Othman et al., 2009). Morphometric and gravimetric parameters of the rice frog living in paddy fields with different degree of herbicide utilization were examined in order to test for influence of herbicide contamination on non-target organisms.

Objective

To examine morphometric and gravimetric parameters of the rice frog living in paddy fields at Nan Province.

Materials and Methods

1. Study Sites

There are a lot of agricultural areas In Nan Province where herbicides have been used for a long time, especially in Wiang Sa District. In this study, two paddy fields in Wiang Sa District, Nan Province, were selected to be study sites including:

1.1 Contaminated Site

The contaminated site (location: 47Q 068772, UTM 2054283) is a paddy field in Lai-nan Sub-District where intensive use of atrazine, glyphosate and paraquat herbicides have been recorded. (Chairaja, interview, October 6, 2009).

1.2 Reference Site

The reference site (location: 47Q 0686779, UTM 2047187) is an organic paddy field in San Sub–District where agrochemicals, including herbicide, was not used for more than 10 years (Kamsrikaew, interview, October 6, 2009).

2. Frog Sample Collection

Frogs were field-collected on monthly basis (July 2010–June 2011) from two paddy fields. Male, female and juvenile frogs were collected by hand at night during visual encounter surveys (Crump and Scott, 1994). The physical factors including temperature, soil pH and relative humidity were recorded during frogs collection.

3. Morphometric and Gravimetric Analyses

After transportation to a laboratory at the Chulalongkorn University Forest and Research Station, Nan Province, frogs were euthanized by immersion into 0.5% MS-222 solution. Snout-vent length (SVL) and body weight (BW) of each frog was recorded, and weight of liver, kidney and gonad (ovary or testis) of each individual were measured after dissection. These organs were preserved in 10% neutral buffer formalin for further study. The body length was measured with Mitutoyo Absolute Digimatic caliper (accuracy 0.01 mm) and body weigth was weighed with Ohaus Pioneer Analytical Balances PA214 (accuracy 0.0001 g).

3.1 Overall Health Status

Frog populations was evaluated for condition factor (CF; Eastwood and Couture, 2002), an indicator of overall health previously used in this frog species (Othman, 2009). 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 (Othman, 2009). The constant *b* or slope of these populations was thus further compared according to Zar (1999)

3.2 Gravimetric Analyses

Gravimetric analysis was used to examine change in status of each organ. Hepatosomatic index (HSI), renosomatic index (RSI), and gonadosomatic index (GSI) were determined from relative weight as followed:

- 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)

4. Statistical Analysis

All parameters were tested for normal distribution and homogeneity of variance. The Scaling coefficient was compared between sites by student's *t* test (Zar, 1999). The condition factor was compared between sites by Mann-Whitney rank sum test. Hepatosomatic, renosomatic and gonadosomatic indices were compared between sites by two-way analysis of variance (ANOVA) using site as a major independent factor and season as a second independent factor. In case of significant difference between sites was found, multiple comparison was carried by Student-Newman-Keuls methods.

The Spearman rank order correlations were used to determine correlation among all morphomertic, gravimetric parameters (condition factor, hepatosomatic index, renosomatic index and gonadosomatic index) and residues of herbicides in frog tissues (Chapter III). Sigma Plot 11.0 was used as a 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 (6.00-7.00), temperature (18-28 °C), relative humidity (73%-96%), 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 weight-length relationship of *F. limnocharis* from contaminated and reference sites are shown in Figure 4.1.

From the regression analyses of relationship between body weight and snout-vent length, the Scaling coefficient of frog in the contaminated site was significantly higher than and those in the reference site (3.06 in reference site vs. 3.20 in contaminated site; $p<0.05$, student's *t* test; Table 4.1), indicating the difference in growth pattern between these two populations.

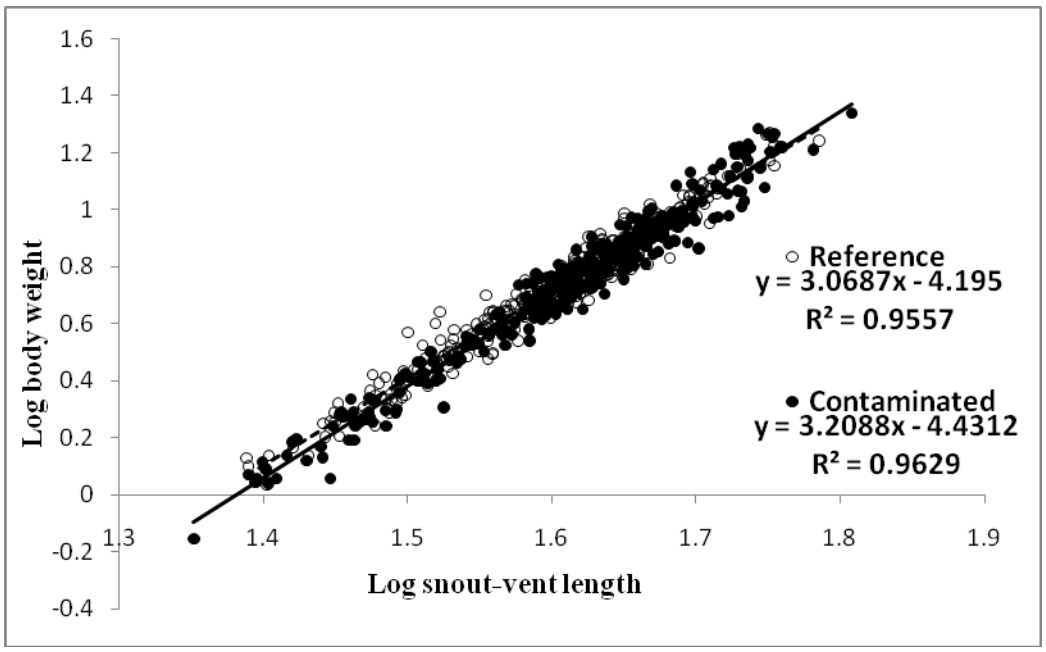


Figure 4.1 Regression analyses of relationship between body weight and snout-vent length of *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Table 4.1 Scaling coefficients of populated rice frog *Fejervarya limnocharis* from reference and contaminated sites at Nan Province, Thailand

	Reference site	Contaminated site
Scaling coefficient	3.06	3.20*

Remark:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (Student's *t*-test, $p<0.05$).

In addition, it was found that the condition factor of frogs in the contaminated site (100.11±0.73) was significantly lower than those in the reference site (104.54±0.70) (two-way ANOVA, $p<0.05$; Table 4.2)

To accommodate statistical analysis, frogs were grouped according to sampling period into 4 groups including July-September 2010 (late wet period), October-December 2010 (early dry period), January-March 2011 (late dry period) and April-June 2011 (early wet period). It was found that the condition factor of rice frog were significantly different between sampling periods in each site. In the reference site, the condition factor in July-September 2010 and April-June 2011 were significantly lower than those in October-December 2010 and January-March 2011 (Table 4.2; 98.37±1.05 and 100.01±1.42 vs 106.02±1.61 and 107.03±1.09, $p<0.05$). In the contaminated site, the condition factor in July-September 2010 was significantly lower than those in October-December 2010, January-March 2011 and April-June 2011 (Table 4.2; 96.09±1.57 vs 101.14±1.06, 103.42±1.81 and 101.82±1.27, $p<0.05$).

Table 4.2 Conditon factors (Mean±SEM) of populated rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/year	Reference site	Contaminated site
late wet	Jul/10 - Sept/10	98.37 ± 1.05[a] (N=82)	96.09 ± 1.57[a] (N=86)
early dry	Oct/10 - Dec/10	106.02 ± 1.61[b] (N=76)	101.14 ± 1.06[b]* (N=86)
late dry	Jan/11 - Mar/11	107.03 ± 1.09[b] (N=60)	103.42 ± 1.81[b] (N=43)
early wet	Apr/11 - Jun/11	100.01 ± 1.42[a] (N=58)	101.82 ± 1.27[b] (N=65)
overall	Jul/10 - Jun/11	104.54 ± 0.70 (N=276)	100.11 ± 0.73* (N=280)

Remarks:

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

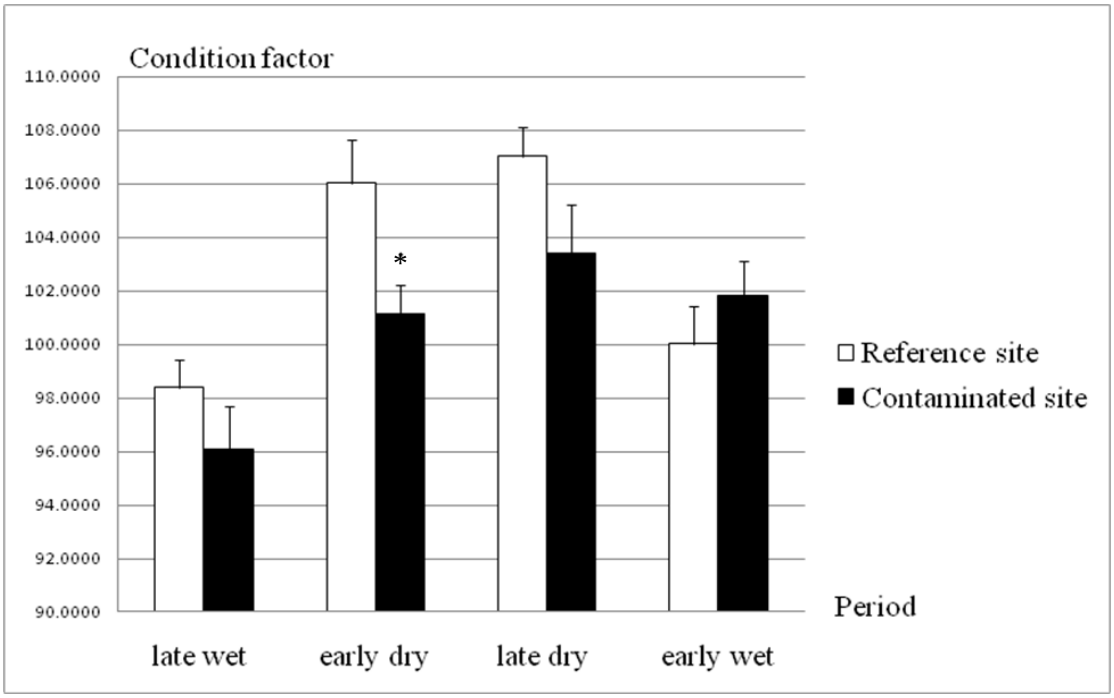


Figure 4.2 Conditon factors (Mean±SEM) of populated rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remark:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (two-way ANOVA, $p<0.05$).

3. Gravimetric Analysis

Gravimetric analysis of frogs by two-way ANOVA showed significant difference between sites as well as between sampling periods in several parameters.

3.1 Hepatosomatic Index (HSI)

The hepatosomatic index was significantly higher in the contaminated site frogs compared to those of the reference site in both male (Table 4.3; overall least square mean: 2.06±0.09 vs. 1.79±0.10; $p<0.05$) and female frogs (Table 4.4; overall least square mean: 2.39±0.07 vs. 2.07±0.07; $p<0.05$).

Table 4.3 Hepatosomatic indices (Mean±SEM) of male rice frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand.

Period	Month/year	Reference site	Contaminated site
late wet	Jul/10 - Sept/10	1.86 ± 0.07 (N=40)	1.92 ± 0.13 (N=24)
early dry	Oct/10 - Dec/10	1.83 ± 0.00 (N=2)	2.17 ± 0.55 (N=3)
late dry	Jan/11 - Mar/11	1.73 ± 0.05 (N=27)	1.97 ± 0.19 (N=12)
early wet	Apr/11 - Jun/11	1.71 ± 0.05 (N=28)	2.16 ± 0.09* (N=32)
overall	Jul/10 - Jun/11	1.78 ± 0.04 (N=97)	2.05 ± 0.07* (N=71)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (two-way ANOVA, $p<0.05$).
- There was no significant difference among sampling periods within the same site (two-way ANOVA, $p>0.05$).

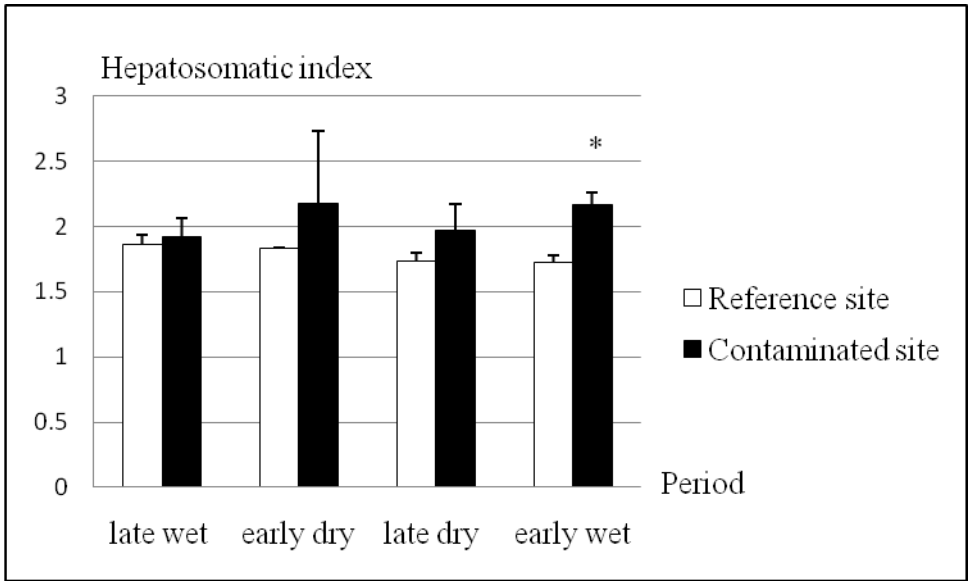


Figure 4.3 Hepatosomatic indices (Mean±SEM) of male frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remark:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (two-way ANOVA, $p<0.05$).

The hepatosomatic index of female frog in the contaminated site was significantly different between sampling periods. The hepatosomatic index in July-September 2010 and October-December 2010 were significantly higher than those in January-March 2011 and April-June 2011(Table 4.4; 2.79±0.13 and 2.76±0.32 vs. 2.09±0.09 and 1.90±0.09, $p<0.05$).

Table 4.4 Hepatosomatic indices (Mean±SEM.) of female rice frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/year	Reference site	Contaminated site
late wet	Jul/10 - Sept/10	1.86 ± 0.08[a] (N=19)	2.79 ± 0.13[a]* (N=34)
early dry	Oct/10 - Dec/10	2.31 ± 0.14[a] (N=13)	2.76 ± 0.32[a] (N=8)
late dry	Jan/11 - Mar/11	1.97 ± 0.11[a] (N=20)	2.09 ± 0.09[b] (N=20)
early wet	Apr/11 - Jun/11	2.12 ± 0.08[a] (N=22)	1.90 ± 0.09[b] (N=28)
overall	Jul/10 - Jun/11	2.05 ± 0.05 (N=74)	2.35 ± 0.08* (N=90)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (two-way ANOVA, $p<0.05$).
- Difference in superscript letter indicates significant difference among sampling periods within the same site (two-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

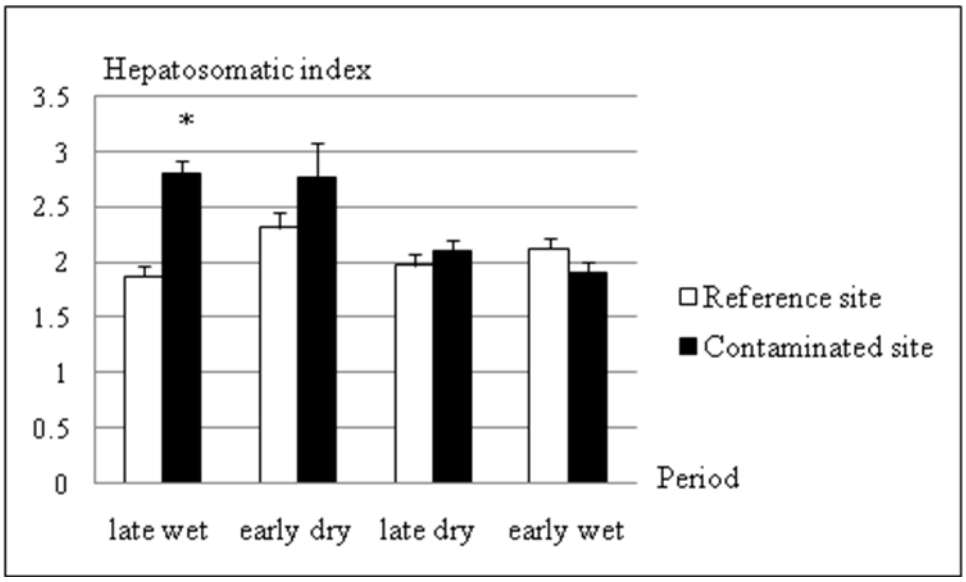


Figure 4.4 Hepatosomatic indices of female frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remark:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (two-way ANOVA, $p<0.05$).

3.2 Renosomatic Index (RSI)

No significant site-related difference in renosomatic index was found in both male (Table 4.5; overall least square mean: contaminated site=0.39$\pm$0.01, reference site=0.39$\pm$0.02, $p>0.05$) and female frogs (Table 4.6; overall least square mean: contaminated site=0.41$\pm$0.01, reference site=0.42$\pm$0.01, $p>0.05$).

Table 4.5 Renosomatic indices (Mean±SEM) of male rice frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/year	Reference site	Contaminated site
late wet	Jul/10 - Sept/10	0.42 ± 0.01 (N=40)	0.38 ± 0.01 (N=24)
early dry	Oct/10 - Dec/10	0.37 ± 0.01 (N=2)	0.36 ± 0.03 (N=3)
late dry	Jan/11 - Mar/11	0.39 ± 0.01 (N=27)	0.41 ± 0.02 (N=12)
early wet	Apr/11 - Jun/11	0.37 ± 0.01 (N=28)	0.40 ± 0.01 (N=32)
over all	Jul/10 - Jun/11	0.40 ± 0.00 (N=97)	0.39 ± 0.00 (N=71)

Remark:

- There was no significant site-related nor seasonal difference (two-way ANOVA, $p>0.05$)

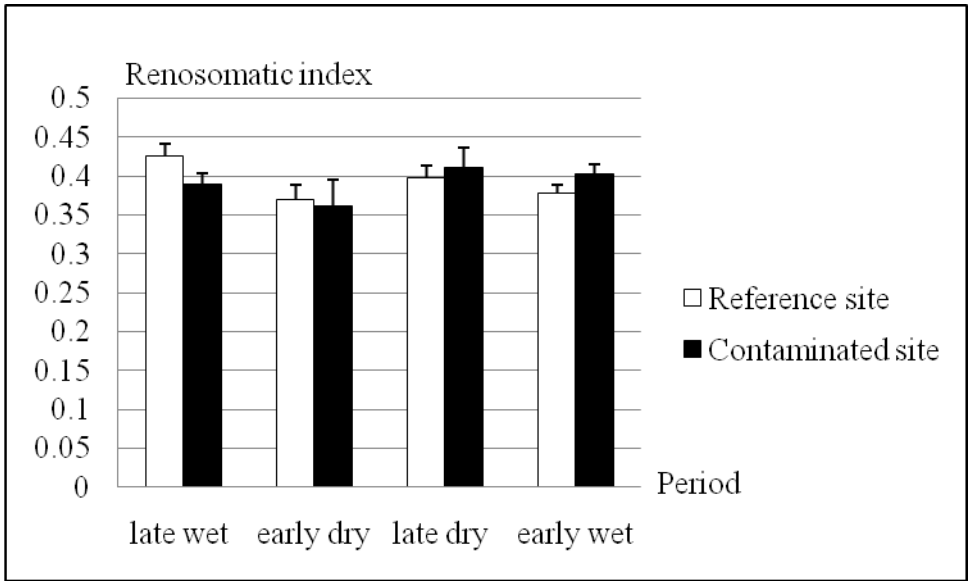


Figure 4.5 Renosomatic indices (Mean±SEM) of male frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remark:

- There was no significant site-related nor seasonal difference (two-way ANOVA, $p>0.05$)

The renosomatic index of female frog was significantly different between sampling periods at each site. In the reference site, the renosomatic index in October-December 2010 was significantly higher than those in January-March 2011 and April-June 2011(Table 4.6; 0.46±0.01vs 0.38±0.01 and 0.37±0.02, $p<0.05$). In the contaminated site, the renosomatic indices in July-September 2010 and October-December 2010 were significantly higher than those in January-March 2011 and April-June 2011 (Table 4.6; 0.45±0.02 and 0.39±0.01 and 0.39±0.01 vs. 0.41±0.01 and 0.37±0.01, $p<0.05$).

Table 4.6 Renosomatic indices (Mean±SEM) of female rice frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/year	Reference site	Contaminated site
late wet	Jul/10 - Sept/10	0.44 ± 0.02[ab] (N=19)	0.45 ± 0.02[a] (N=34)
early dry	Oct/10 - Dec/10	0.46 ± 0.01[a] (N=13)	0.39 ± 0.01[a] (N=8)
late dry	Jan/11 - Mar/11	0.38 ± 0.01[b] (N=20)	0.41 ± 0.01[b] (N=20)
early wet	Apr/11 - Jun/11	0.37 ± 0.02[b] (N=22)	0.37 ± 0.01[b] (N=28)
over all	Jul/10 - Jun/11	0.41 ± 0.01 (N=74)	0.41 ± 0.01 (N=90)

Remarks:

- There was no significant difference between sites (two-way ANOVA, $p>0.05$).
- Difference in superscript letter indicates significant difference among sampling periods within the same site (two-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

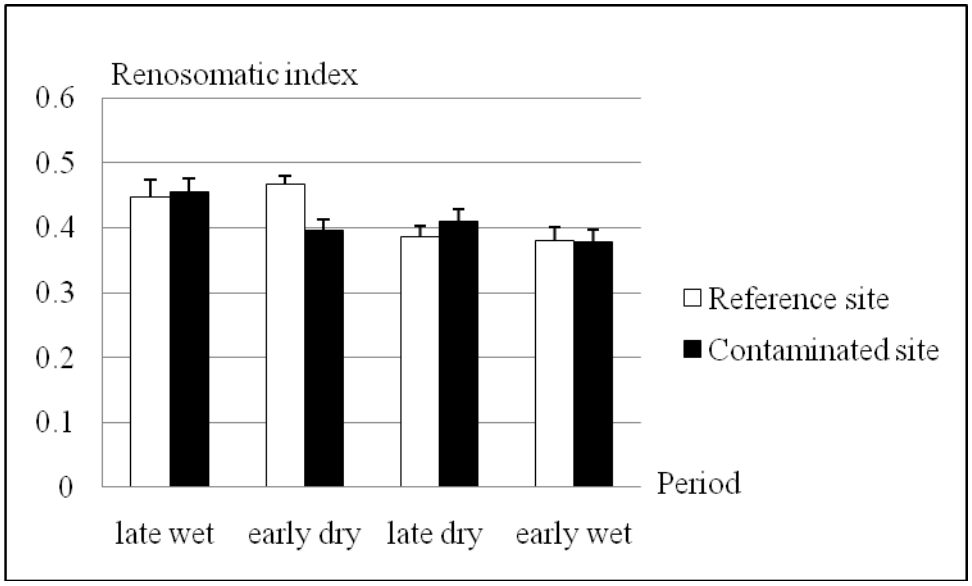


Figure 4.6 Renosomatic indices (Mean±SEM) of female frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remark:

- There was no significant difference between sites (two-way ANOVA, $p>0.05$).

3.3 Gonadosomatic Index (GSI)

No significant site-related difference in the relative testicular weight (Table 4.7; overall least square mean: contaminated site=0.23±0.01, reference site=0.23±0.01, $p>0.05$) was found in these frog populations. However, the gonadosomatic index of female frogs (ovarian weight, Table 4.8) in the contaminated site was significantly higher than those of the reference site frogs (overall least square mean: 8.03±0.70 vs. 5.48±0.68; $p< 0.05$).

The gonadosomatic index of male frog was significantly different between sampling periods at each site. In the reference site, the gonadosomatic indices in July-September 2010 and April-June 2011 were significantly higher than that in January-March 2011 (Table 4.7; 0.25±0.01 and 0.24±0.00 vs. 0.19±0.01, $p<0.05$). In the contaminated site, the gonadosomatic index in July-September 2010 was significantly higher than those in October-December 2010 and January-March 2011 (Table 4.7; 0.27±0.01 vs. 0.18±0.02 and 0.19±0.01, $p<0.05$).

Table 4.7 Gonadosomatic indices (Mean±SEM) of male rice frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Period	Month/year	Reference site	Contaminated site
late wet	Jul/10 - Sept/10	0.25 ± 0.01[a] (N=40)	0.27 ± 0.01[a] (N=24)
early dry	Oct/10 - Dec/10	0.21 ± 0.04[ab] (N=2)	0.18 ± 0.02[b] (N=3)
late dry	Jan/11 - Mar/11	0.19 ± 0.01[b] (N=27)	0.19 ± 0.01[b] (N=12)
early wet	Apr/11 - Jun/11	0.24 ± 0.00[a] (N=28)	0.26 ± 0.00[ab] (N=32)
over all	Jul/10 - Jun/11	0.23 ± 0.00 (N=97)	0.25 ± 0.00 (N=71)

Remark:

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

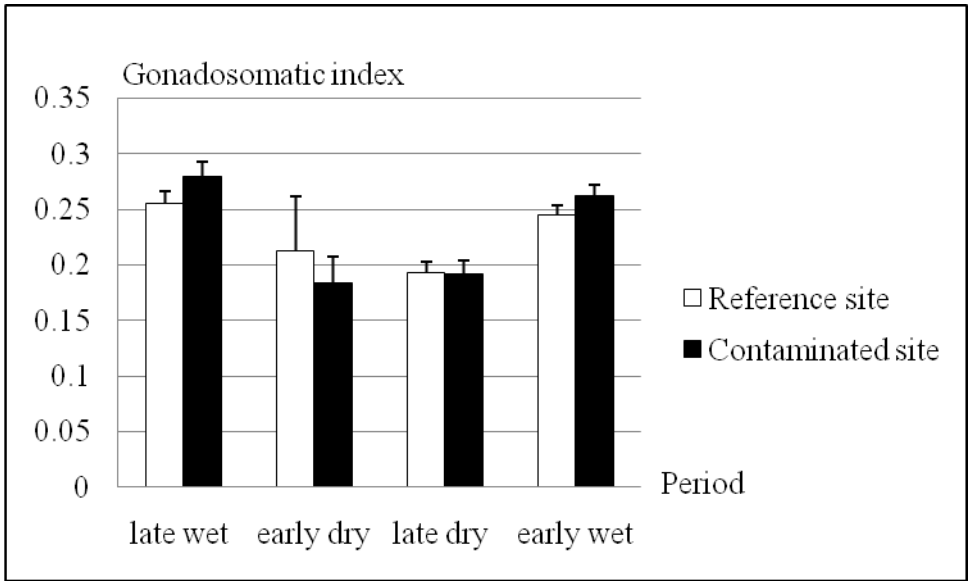


Figure 4.7 Gonadosomatic indices (Mean±SEM) of male frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remark:

- There was no significant difference between sites (two-way ANOVA, $p>0.05$).

The gonadosomatic index of female frog was significant different between sampling periods at each site. In the reference site, the gonadosomatic indices in July-September 2010, October-December 2010 and January-March 2011 were significantly lower than that in April-June 2011 (Table 4.8; 4.54±1.04, 0.62±0.06 and 4.30±0.65 vs. 12.42±1.60, $p<0.05$). In the contaminated site, the gonadosomatic indices in July-September 2010 and April-June 2011 were significantly higher than those in October-December 2010 and January-March 2011 (Table 4.8; 12.13±1.25 and 12.67±1.21 vs. 1.64±1.00 and 5.67±1.06, $p<0.05$).

Table 4.8 Gonadosomatic indices (Mean±SEM) of female rice frogs *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand.

Period	Month/year	Reference site	Contaminated site
late wet	Jul/10 - Sept/10	4.54 ± 1.04[a] (N=19)	12.13 ± 1.25[a]* (N=34)
early dry	Oct/10 - Dec/10	0.62 ± 0.06[a] (N=13)	1.64 ± 1.00[b] (N=8)
late dry	Jan/11 - Mar/11	4.30 ± 0.65[a] (N=20)	5.67 ± 1.06[b] (N=20)
early wet	Apr/11 - Jun/11	12.42 ± 1.60[b] (N=22)	12.67 ± 1.21[a] (N=28)
over all	Jul/10 - Jun/11	6.13 ± 0.75 (N=74)	9.93 ± 0.76* (N=90)

Remarks:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (two-way ANOVA, $p<0.05$).
- Difference in superscript letter indicates significant difference among sampling periods within the same site (two-way ANOVA, $p<0.05$; followed by Student-Newman-Keuls' multiple comparison).

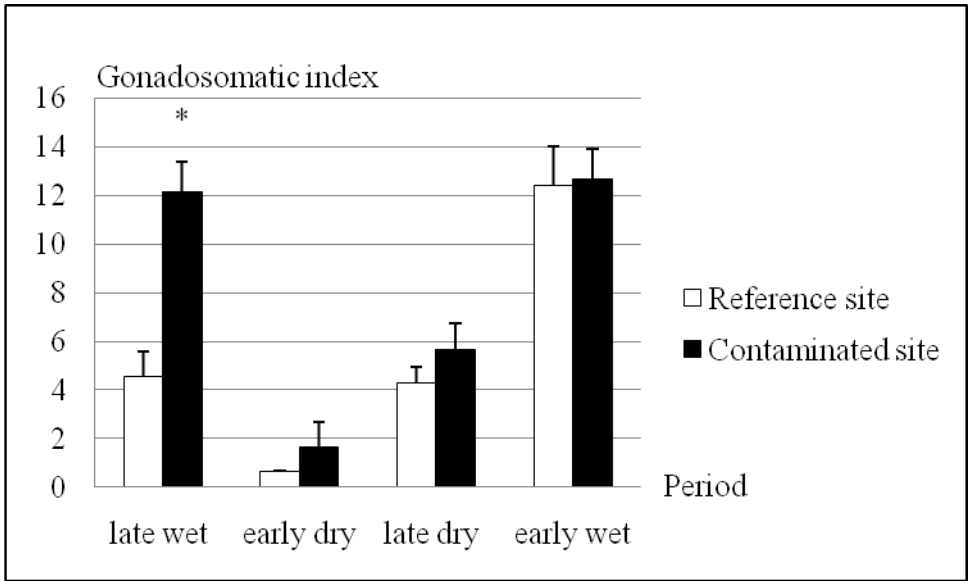


Figure 4.8 Gonadosomatic indices of female frogs *Fejervarya limnocharis* from reference and contaminated site paddy fields Nan Province, Thailand

Remark:

- An asterisk (*) indicates significant difference between the contaminated site and the reference site (two-way ANOVA, $p<0.05$).

4. Correlation Analysis

The results of Spearman Product Moment Correlation showed a significant correlation only between glyphosate and paraquat residues in tissue of the rice frog. There was no significant correlation among atrazine, glyphosate and paraquat residues in frog tissue and any morphometric or gravimetric parameters (condition factor, hepatosomatic index, renosomatic index and gonadosomatic index; Table 4.9).

Table 4.9 The Spearman rank order correlations of herbicide contamination in frog tissues and morphometric and gravimetric parameters

Parameters		Correlation coefficient (r)	P value
Atrazine vs.	Glyphosate (N=44)	-0.102	0.507
	Paraquat (N=43)	-0.0520	0.739
	Condition factor (N=48)	-0.0330	0.823
	Hepatosomatic index (N=16)	0.241	0.360
	Renosomatic index (N=16)	0.356	0.171
	Gonadosomatic index (testis) (N=8)	-0.333	0.387
	Gonadosomatic index (ovary) (N=8)	0.333	0.387
Glyphosate vs.	Paraquat (N=43)	0.332	0.0299*
	Condition factor (N=44)	-0.111	0.477
	Hepatosomatic index (N=16)	0.050	0.848
	Renosomatic index (N=16)	-0.382	0.139
	Gonadosomatic index (testis) (N=8)	-0.119	0.749
	Gonadosomatic index (ovary) (N=8)	-0.381	0.321
Paraquat vs.	Condition factor (N=44)	-0.0761	0.622
	Hepatosomatic index (N=16)	0.103	0.697
	Renosomatic index (N=16)	-0.115	0.664
	Gonadosomatic index (testis) (N=8)	-0.262	0.498
	Gonadosomatic index (ovary) (N=8)	-0.571	0.120

Remark:

- An asterisk (*) indicates a significant correlation.

Discussion

The Scaling coefficient of frogs in contaminated site was different from those in the reference site indicating different growth pattern between these two populations of frogs living in areas with different degree of herbicide utilization. These findings agree with several prior reports in which herbicides, especially atrazine, were believed to disturb development and growth pattern of amphibians (Hayes et al., 2006; Storrs and Semlitsch, 2008; Spear et al., 2009; Lenkowski et al., 2010).

In addition, the difference in condition factor or an indicator of overall health status illustrated 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. Since the smaller frogs could be easily captured as a prey in food chain (Hayes et al., 2006), the CF could indicate lower fitness of frogs living in the contaminated area. Since other factors such as climate, geography, agricultural activities and physical factors are similar between these two sites, it seems that utilization of herbicides could affect overall health of the frogs. This is supported by previously published articles in which herbicides utilization was found to increase environmental stressors (Soderman et al., 2007), cause toxic effect to the frog (Lajmanovich et al., 2010) or disrupt steroidogenesis and growth hormone secretion (Hayes et al., 2006).

Significant differences of condition factor between periods in each site of this study were different from the studies in other frog species. Jelodar and Fazli (2012) studied the condition factor of *Rana ridibunda* collected during July-September 2010 from the North Iran where metal contamination was observed. The result showed that condition factor of frog was not significantly different amoung months. This could be due to a relatively shorter study period (3 months) in *Rana ridibunda* compared to a year round study in *Fejervarya limnocharis*. Normally the rice frog in paddy fields could undergo aestivation when their food (insect) was decrease (Toshiaki and Masafumi, 2001). As a result, seasonal fluctuating in condition factor could be due to different in food availability within a year.

The hepatosomatic indices (HSI) were significantly higher in the contaminated site frogs compared to those in the reference site for both male and female. Since liver is the main target organ for accumulation and detoxification of xenobiotics (Crawshaw and Weinkle, 2000), it is highly probable that frogs lived in areas with

intensive herbicides usage would show enlarge liver as a sign of exposure to and accumulation of xenobiotic contaminants. Evidence of detoxification activity was shown in report by Parvez and Raisuddin (2006) that herbicide paraquat can reduce glutathione levels in liver of *Channa punctata* fish so that the liver have to work harder in order to eliminate this herbicide and other contaminant. The similar finding in the same species of frog was reported by Othman (2009) when the rice frogs lived in cadmium contaminated agricultural areas tended to have larger and heavier liver as a result of xenobiotic exposure.

Interestingly, the hepatosomatic index of female frog from the contaminated site was significantly different between sampling periods. HSI in wet season was significantly higher than those in the dry season. This could be related to different level of herbicide contamination in frog tissue. The highest concentration of atrazine in frog tissue was found in July 2010 (wet season), corresponding to the higher HSI in the frogs from the contaminated site. It is of interest to note that seasonal difference in hepatosomatic index was not found in the reference site animal.

No significant site-related difference in renosomatic index (RSI) was found in both male and female frogs. It is possible that kidney is not the main target organ for accumulation or detoxification of these herbicides. In addition, significant seasonal difference in renosomatic index of the female frog was evidenced in both reference and contaminated site, possibly due to the seasonal fluctuating in kidney weight of this frog species.

No significant site-related difference in the relative testicular weight was found in these frog populations, partly due to high individual variation and small sample size. However, the gonadosomatic index of female frogs in contaminated site was significantly higher than those of the reference site frogs. The larger ovary in contaminated site frog is possibly due to effects of herbicides on ovarian growth. Herbicide used in agricultural area of Nan Province, especially atrazine, is a known endocrine-disrupting chemical (Kavlock, 2001; McKinlay et al., 2008) that can exert several estrogenic effects (Hayes et al., 2002, 2006, 2010; Kloas and Lutz, 2006). Hayes et al. (2002) also reported that the African clawed frogs *Xenopus laevis* exposed to atrazine at level as low as 0.1 ppb (0.1 µg/L) can show hermaphroditism in, and level as low as 1 ppb (1 µg/L) can reduce laryngeal size in the male frog.

Langlois et al. (2010) reported that low level of atrazine can affect on sex ratio of *Rana pipiens* by increase number of female frog. It was also reported that atrazine could induce male-to-female sex reversal in *X. laevis* and the resultant female frogs could produce viable eggs (Hayes et al., 2010).

Interestingly, most of the previous report on estrogenic effects of atrazine focused on effect on male frog, with few study on female frogs. This study is one of a rare evidence of potential effect of atrazine on reproductive system of the female frog. Estrogen is a gatekeeper of female reproductive health (McLachlan et al., 2006), therefore contaminant with estrogenic effect should be as effective to some extent. 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. In this study, gross anatomy examination revealed that the heavier ovary with mature eggs in the contaminated site frog was found even in dry period when egg laying was not possible since every 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.

Since conflicting results on estrogenic effects of atrazine are also evident in many studies. Murphy et al. (2006) suggested that an alteration in reproductive system was a natural process and ovotestis could be found in both atrazine contaminated site and reference site. Other studies also showed that weak support was found on the estrogenic effect of atrazine in *X. laevis* (Coady et al., 2005; Oka et al., 2008). One may argue that reproductive effects of atrazine were still uncertain. However, other herbicides used in this area also showed potential reproductive effects. Paraquat and glyphosate were found to have impacts on reproductive system of *Rana esculenta* (Quassinti et al., 2009). Glyphosate alone was also toxic to tadpole of *Rhinella arenarum* (Lajmanovich et al., 2010) and could increase stress in many frog species (Relyea, 2005). Since pesticides are known to cause adverse effect in synergistic fashion (Hayes et al., 2006), mixture of herbicides used in the agricultural areas of Nan Province may also act in similar ways.

As expected, the results of gonadosomatic index of male and female frog at both sites showed a significant seasonal difference, confirming that the rice frog is the seasonal breeder with the breeding season in rainy season or wet period. Change in

testicular weight and ovarian corresponds with reproductive activities of this frog species and in accordance with local seasonal patterns (Othman et al., 2011).

Correlative study revealed only a significant association between glyphosate residues and paraquat residue in frog tissue. The correlation may indicate a pattern of herbicide mixture used in this area where these two herbicides were used in concurrent. As a results, frog exposing to the glyphosate would also have chance to exposeto paraquat as well. Correlations of herbicides contamination and all morphometric and gravimetric parameters were not significant. This could be due to the fact that the compromised morphometric and gravimetric parameters were caused by synergistic effects of various contaminants (not a single herbicide) and other factors in the real environment (Hayes et al., 2006; Govindarajulu, 2008).

Conclusion

Using the rice frog as a sentinels for herbicide contamination, the result of condition factor indicated that the overall health status of frog was decreased in the paddy field where herbicide was intensively used. In addition, hepatosomatic index and gonadosomatic index indicated that frog from the contaminated site has enlarged liver and enhanced ovarian growth even in the dry period. 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.

CHAPTER V

INFLUENCE OF HERBICIDES ON POPULATION OF RICE FROG *Fejervarya limnocharis* IN PADDY FIELDS, NAN PROVINCE

Introduction

Chemical contamination has become one of the global environmental problems for many decades. The contamination could come from wide array of source such as industrial, urban and farming activities. The contamination not only affects human well-being, but also becomes an environmental stressor for ecosystem health (Chang et al., 2007). In agricultural area, the used of pesticide is regarded as one of the major source of environmental contamination since the pesticides have a limit capability to be degraded in nature. Upon an intensive and continuous use of pesticides, their residues are likely to be present in the agricultural area. This contamination can lead to an exposure to non-target organisms living in the agricultural areas as well as increased in environmental stress (Chang et al., 2007).

The environmental stress from pesticide contamination may increase in any vertebrates living in vicinity of the agricultural areas. Since the vertebrates share several similar structure and function of their organs to human, monitoring an increase in environmental stress induced by pesticide contamination could be used as early warning for human population residing in the affected areas as well.

Amphibian is regarded as one of a suitable sentinel species for environmental stress induced by herbicide contamination. Their natural habitats include agricultural areas where they can be exposed to pesticides (Khan and Law, 2005). Their existence both in water and on land makes them susceptible to pesticide exposure through several routes, especially through their semi-permeable skin (Duellman and Trueb, 1994; Roy, 2002; Quaranta et al., 2009).

Pesticide contamination in an agricultural area may affect morphology as well as growth rate of each individual frog and age structure of the frog population. Soderman et al. (2007) reported that environmental stress can influence on growth rate of the moor frog *Rana arvalis*. Therefore, in an area with intensive and continuous herbicide utilization, the study on age structure of the amphibian

population could be used as a tool for evaluating impact of environmental stress induced by herbicide contamination.

To determine age of individual amphibian, a skeletochronological technique has been regarded as a suitable method for natural population of amphibians and reptiles (Hemelaar, 1988). This method is based on the fact that an animal living in nature would have a period of slow or arrested growth in certain time of the year. This period corresponded with a presence of line of arrested growth (LAG) in a cross section of long bone on one line one year basis. It is become the popular method for study the age of amphibians, especially in the temperate species (Kusano et al., 1995). Khonsue et al. (2000) indicated that the skeletochronology can be used for age determination in a tropical frog *Rana nigrovitta* in Thailand. Therefore, it is interesting to apply this technique to the rice frog in order to see whether frogs from these two sites would have difference in age structure.

To monitor effects of stressors on vertebrate population, a fluctuating asymmetry (FA), or an organism's deviation from perfect bilateral symmetry (Palmer, 1994), has been successfully used as an effective biomarker. Environmental stress is regarded as a contributing factor toward developmental instability or developmemtal noise that could make the organism to show higher degree of FA (Palmer and Strobeck, 1986; 2003). Moller and Swaddle (1997) reviewed and indicated that several studies have shown a relationship between FA and environmental stress in population level of organisms. The studies on fluctuating asymmetry of amphibian can thus be used to evaluate the environmental stress on amphibians (Soderman et al., 2007 and Helle et al., 2011), and could possibly be used to monitor the long-term impact of herbicide contamination on amphibian population.

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). Fluctuating asymmetry analysis and skeletocronology of the populated rice frog in paddy fields with different degree of herbicide utilization were examined in order to test for influence of herbicide contamination on population level of the non-target organisms.

Objectives

1. To determine and compare age structure of the rice frog population between reference paddy field and contaminated paddy field.
2. To compare fluctuating asymmetry of the populated rice frog between reference paddy field and contaminated paddy field.

Materials and Methods

1. Study Sites

There are a lot of paddy fields in Wiang Sa District, central part of Nan Province, where herbicide mixtures have been used extensively for long periods. The rice frog living in these paddy fields is thus susceptible to prolonged exposure to environmental stress from herbicide contamination. In this study, two paddy fields in Wiang Sa District were chosen to be used as study sites.

1.1 Contaminated Site

A paddy field in Lai-nan Sub-District (location: 47Q 068772, UTM 2054283) where intensive herbicide utilization hase been reported (Chairaja, interview, October 6, 2009) was selected as a potential contaminated site.

1.2 Reference Site

An organic paddy field with no history of agrochemicals usage (Kamsrikaew, interview, October 6, 2009) in San Sub-District (location: 47Q 0686779, UTM 2047187) was selected as a reference site in this study.

2. Frog Sample Collection

Frogs were field-collected during July 2010 to June 2011 from two paddy fields. Male and female frogs were collected by hand at night during visual encounter surveys (Crump and Scott, 1994) on monthly basis. Frog samples were transported to the laboratory at the Chulalongkorn University Forest and Research Station, Nan Province for further study.

3. Skeletochronology

After transported to the laboratory, the frogs were euthanized by immersion into 0.5% MS-222 solution. Afterward, phalange of the third digit of the left forelimb was clipped and preserved in 10 % neutral buffered formalin for further tissue preparation.

The fixed phalange was washed in running tap water for 24 hours before proceeding with decalcification in 5 % nitric acid for 30 minutes and washing in running tap water for another 24 hours. The decalcified phalanges were kept in a refrigerator at 4 °C until further tissue sectioning steps.

The phalange was cross-sectioned at 18-20 µm thickness by a freezing microtome (Model 08-150-0; Erma Inc.). After that, the sections were stained with hematoxylin (Mayer's acid hemalum) for 30 minutes and rinsed in tap water for 30 minutes. The stained sections were mounted with glycerin jelly and examined for lines of arrested growth (LAGs) under a light microscope.

4. Preparation of Frog Bones

After euthanization in 0.5% MS-222 solution, snout-vent length (SVL) and body weight (BW) of each frog was recorded, and frogs were dissected for further studies (Chapter IV). The body length was measured by Mitutoyo Absolute Digimatic caliper (accuracy 0.01 mm) and the weight was measured with Ohaus Pioneer Analytical Balances PA214 (accuracy 0.0001 g).

After the dissection, carcass of each individual frog was boiled at 100 °C for 20 minutes. Soft tissue was removed from the skeleton with an aid of forceps and soft bristle toothbrush. Afterward, the appendage bones of forelimb and hindlimb including radio-ulna, humerus, femur and tibio-fibula was individually collected from left side and right side of each frog. These bones were rinse in tap water and kept in hot air oven at 60 °C for 24 hours. Finally, the bones were kept at room temperature in a plastic container with silica gel until further analysis.

5. Frog Bone Measurement

After bone preparation, each bone was measured for length with a micrometer (Moore and Wright; accuracy 0.001 mm), and weight with a digital balance (Chyo;

accurary 0.0001 g). After every bone was measured, the measurement for length and weight was repeated again for the second time in order to account for a potential measurement error.

6. FA Statistical Analysis

A step-by-step flow chart of FA analysis is illustrated in Figure 5.1 and elaborated as followed. In order to determine a fluctuating asymmetry in each population, The following three factors contributing to trait difference were needed to take into consideration (Palmer, 1994) including 1) directional asymmetry (DA), 2) individual variation and 3) nondirectional asymmetry (or fluctuating asymmetry; FA). To account for these factors, two-way analysis of variance (two-way ANOVA) was performed. Side variation (left and right) was used as the first factor, and individual variation was used as the second factor, while the dependent variable was bone weight or bone length. After two-way ANOVA, p-value of an interaction (side variation x individual variation) at 0.05 or below is used as a criteria to indicate that this population has a fluctuating asymmetry (Table 5.1). Upon meeting this requirement, FA10 (Palmer, 1994) of each population was calculated using this formula:

Fa10 = (mean square of interaction (side x individual)) - mean square of residual)/2

To compare and test for significant site-related difference in FA10 between populations of frogs from reference and contaminated sites, the degree of freedom of FA10 (Palmer, 1994) was calculated, and the Fisher-Snedecor distribution was used to optain the p value. All of these calculations were performed on R program using a script written by Dr. Julien Claude (personal communications).

Table 5.1 Source of variation in two-way ANOVA (Palmer, 1994)

Source of variations	Indicator of
Side variation	Directional asymmetry
Individual variation	Size/shape variation
Interaction (Side x Individual)	Non directional asymmetry (Fluctuating asymmetry)

In some certain case, two-way ANOVA may indicate a significant directional asymmetry by showing p-value of side variation at 0.05 or below. In this case, additional analysis is needed to verify that the directional asymmetry was not interfered with the fluctuating asymmetry. Otherwise, the trait with significant DA must be excluded from FA analysis (Palmer, 1994). Palmer and Strobeck (2003) recommended that if the directional asymmetry is less than FA4a value, it means that the DA is so small and unlikely to confound interpretation of the FA. In this study, the DA of each population was calculated from the mean value of difference between R (right side value) and L (left side value), as in the following equation:

Directional Asymmetry (DA) = mean (R-L)

In addition, FA4a of each population was calculated from this formula (Palmer and Strobeck, 2003):

$$\mathrm{FA4a} = 0.798 \times \sqrt{\text{variance R} - \text{L}}$$

Afterward, the DA and FA4a was compared and determined whether the aforementioned assumption of the FA is met.

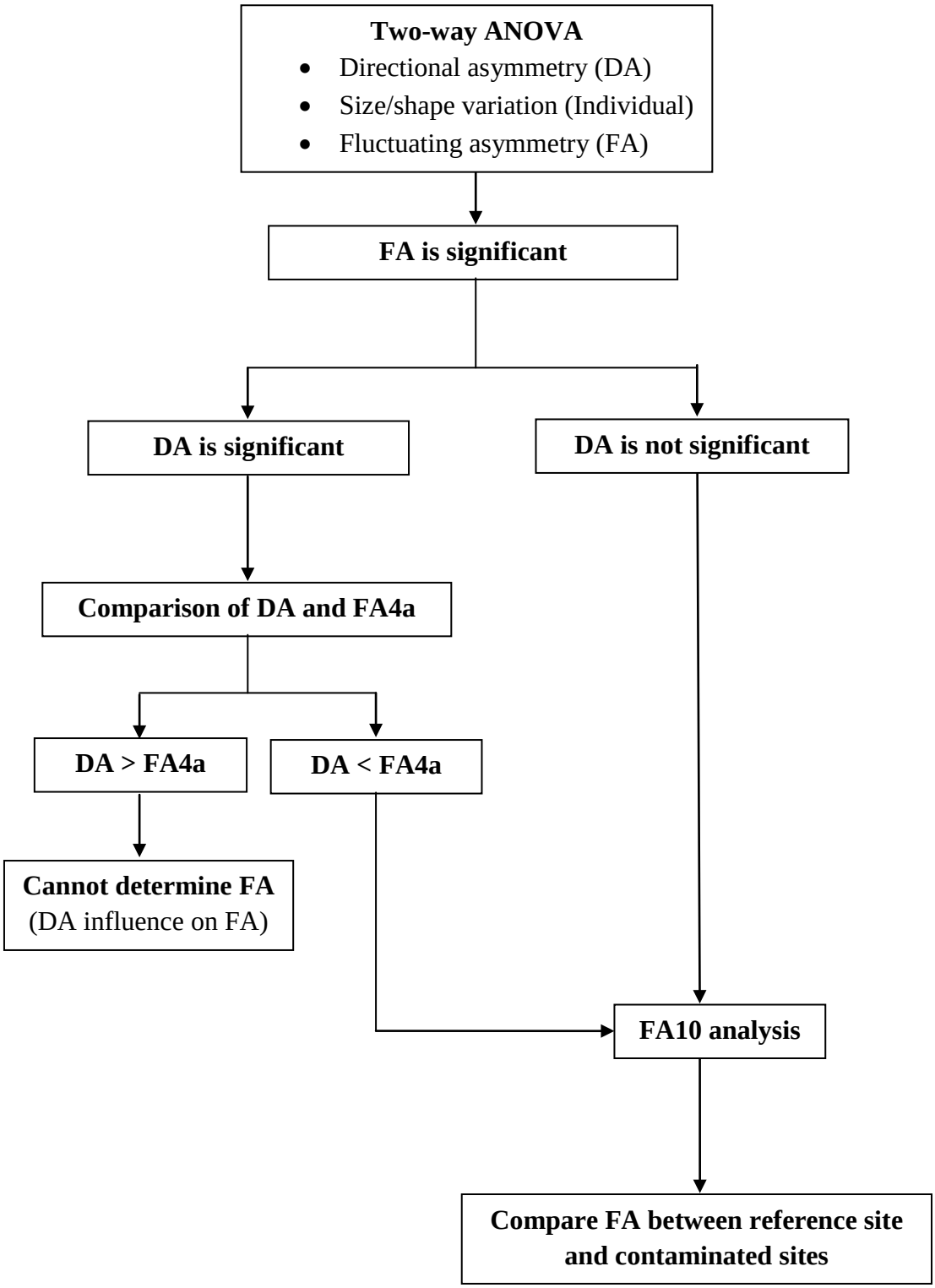


Figure 5.1 Flowchart of fluctuating asymmetry analysis (modified from Palmer, 1994; Palmer and Strobeck, 2003)

Results

1. Skeletochronology

The result of skeletochronological technique showed that the visible lines of arrested growth (LAGs) were found in only small number of individuals (29 out of 332 specimens; Figure 5.3). In the reference site, LAGs were found in 18 samples in a range of 1-3 LAGs. In the contaminated site, however, only 1 LAG was found in each of the 11 samples showing LAG in the bone section. Majority of animals (303 specimens) showed very faint lines or no line at all (Figure 5.2).

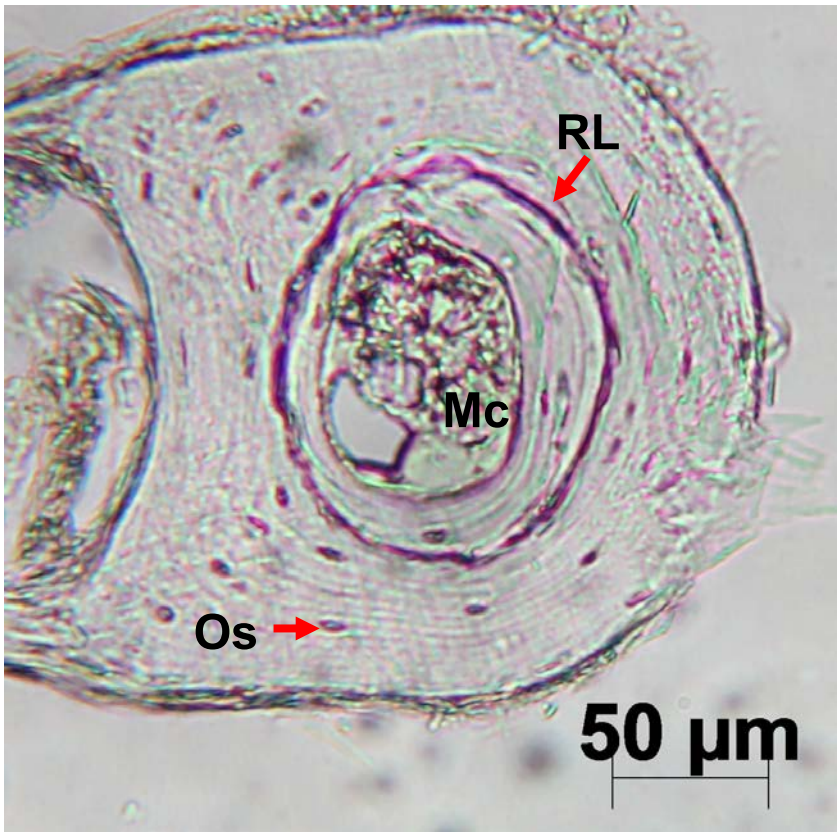


Figure 5.2 A representative micrograph of cross-section of phalange bone of the rice frog *Fejervarya limnocharis* caught from paddy fields at Nan Province, Thailand. Line of arrested growth (LAG) was not found in this section. Mc = marrow cavity, Os = osteocyte, RL = resorption line

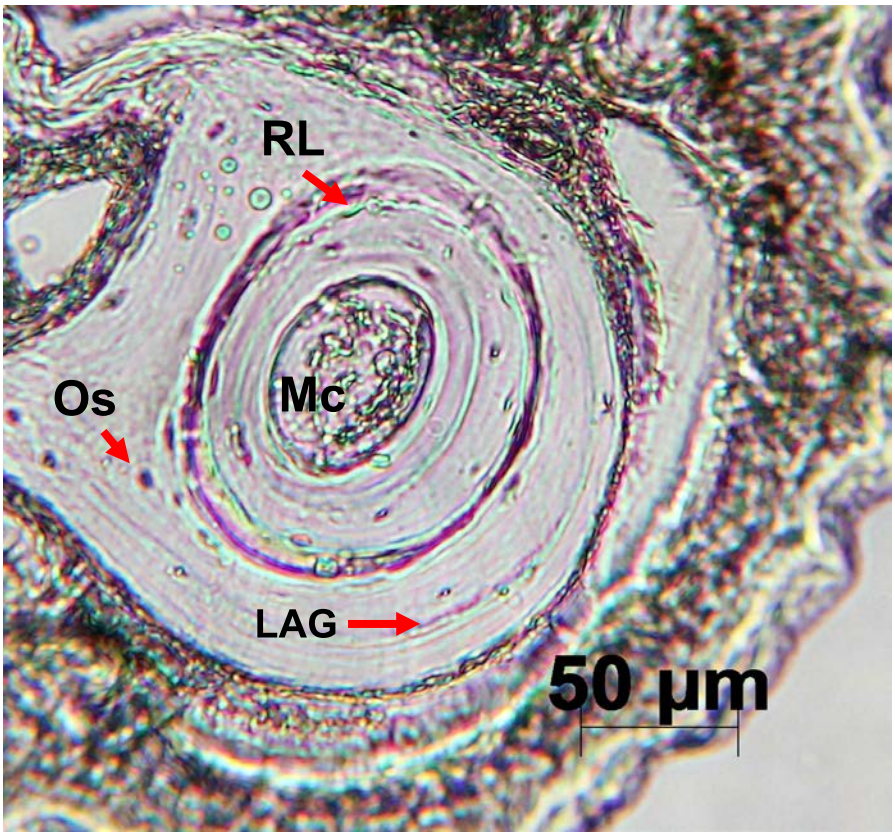


Figure 5.3 A representative micrograph of cross-section of phalange bone of the rice frog *Fejervarya limnocharis* caught from paddy fields at Nan Province, Thailand. Line of arrested growth (LAG) was evidenced in this section. Mc = marrow cavity, Os = osteocyte, RL = resorption line, LAG = line of arrested growth

2. Fluctuating Asymmetry of Male Bone Length

2.1 Two-way ANOVA to Test for FA Assumption

Male Radioulna Length

The results of two–way ANOVA showed that the side variation was not significant in both reference site and contaminated site animals, suggesting that there was no directional asymmetry (DA) in these populations. The individual variation was significant and indicated size/shape variation of frogs from both sites. Of importance to this study, the interaction between side and individual was significant in both populations, meaning that the fluctuating asymmetry (FA) was present in these populations

Table 5.2 Two-way ANOVA of male radioulna length

	Reference site (N=84)		Contaminated site (N=68)	
	Mean square	p-value	Mean square	p-value
Side variation	2.09×10^{-2}	0.07	5.10×10^{-4}	0.82
Individual variation	1.98	< 0.05*	0.86	< 0.05*
Interaction (sid. x ind.)	3.15×10^{-2}	< 0.05*	4.15×10^{-2}	< 0.05*
residual	6.52×10^{-3}	-	1.01×10^{-2}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Male Humerous Length

The results of two–way ANOVA showed that the side variation in humerous length was significant in both populations, suggesting that the directional asymmetry (DA) was existed. Comparison of DA and FA4a showed that the DA was lower than FA4a in both populations, indicating no interference of the DA on FA (Palmer and Strobeck, 2003). The individual variation was significant in both populations, meaning that size/shape variation was evidence in these frogs. Finally, the side and individual interaction was significant in these two populations, suggesting a presence of the fluctuating asymmetry**.**

Table 5.3 Two-way ANOVA of male humerous length

	Reference site (N=82)		Contaminated site (N=68)	
	Mean square	p-value	Mean square	p-value
Side variation	1.50×10^{-1}	< 0.05*	2.00×10^{-2}	< 0.05*
Individual variation	1.53	< 0.05*	3.26	< 0.05*
Interaction (sid. x ind.)	3.74×10^{-2}	< 0.05*	1.09×10^{-2}	< 0.05*
residual	4.71×10^{-3}	-	2.9×10^{-3}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Male Tibiofibula Length

Two–way ANOVA of tibiofibula length showed that the side variation in the reference site frog was not significant, suggesting that there was no directional asymmetry in this population. However, the side variation in contaminated site frog was significant, indicating the presence of directional asymmetry in frogs from this site. However, DA of the contaminated site was lower than FA4a value, suggesting that the DA was not influenced on FA in the contaminated site animals. The individual variation, or an indicative of size/shape variation, was significant in these populations. Similarly, the side and individual interaction was significant in these frogs, indicating that the FA was present in both populations**.**

Table 5.4 Two-way ANOVA of male tibiofibula length

	Reference site (N=91)		Contaminated site (N=66)	
	Mean square	p-value	Mean square	p-value
Side variation	1.10×10^{-3}	0.58	2.75×10^{-2}	< 0.05*
Individual variation	16.71	< 0.05*	9.39	< 0.05*
Interaction (sid. x ind.)	1.15×10^{-2}	< 0.05*	3.37×10^{-2}	< 0.05*
residual	3.70×10^{-3}	-	5.00×10^{-3}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Male Femur Length

The results of two–way ANOVA showed no side variation or directional asymmetry in these populations. However, significances in the individual variation and the interaction between side and individual was evidenced in both populations, suggesting a presence of the fluctuating asymmetry**.**

Table 5.5 Two-way ANOVA of male femur length

	Reference site (N=89)		Contaminated site (N=70)	
	Mean square	p-value	Mean square	p-value
Side variation	3.7×10^{-3}	0.33	1.47×10^{-2}	0.09
Individual variation	10.43	< 0.05*	4.28	< 0.05*
Interaction (sid. x ind.)	8.60×10^{-3}	< 0.05*	3.11×10^{-2}	< 0.05*
residual	4.00×10^{-3}	-	5.20×10^{-3}	-

Remark: An asterisk (*) indicates significant variation or interaction.

2.2 Comparison of FA in Male Bone Length between Sites

After two-way ANOVA, FA10 of each population was calculated according to Palmer (1994) and shown in Table 5.6. Comparisons of FA10 in both reference and contaminated sites revealed that the FA of humerous, tibiofibula and femur in male frogs from the contaminated site were significantly higher than those of the reference site (Table 5.6).

Table 5.6 Fluctuating asymmetry of bone length of male rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

male-bone	Reference site		Contaminated site		F	p-value
	Df	FA	Df	FA		
radioulna	51.19	0.0125 (N=84)	37.10	0.0157 (N=68)	1.26	0.22
humerous	41.37	0.0040 (N=82)	50.81	0.0164* (N=68)	4.10	< 0.05
tibiofibula	45.85	0.0039 (N=91)	45.85	0.0143* (N=66)	3.67	< 0.05
femur	23.07	0.0023 (N=89)	46.47	0.0130* (N=70)	5.65	< 0.05

Remark: An asterisk (*) indicates significant difference from the reference site.

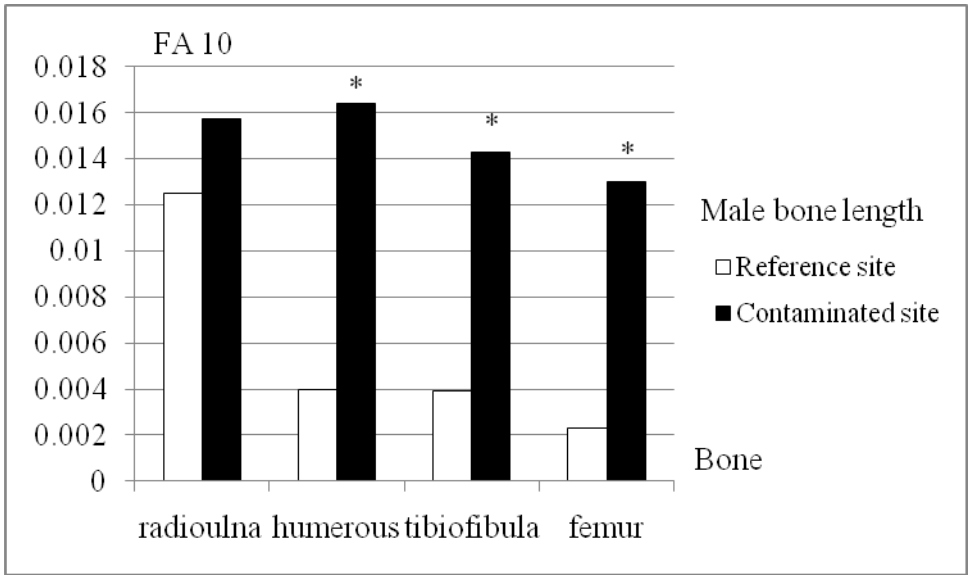


Figure 5.4 Fluctuating asymmetry of bone length of male rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remark: An asterisk (*) indicates significant difference from the reference site.

3. Fluctuating Asymmetry of Male Bone Weight

3.1 Two-way ANOVA to Test for FA Assumption

Male Radioulna Weight

The results of two–way ANOVA show that the side variation in the contaminated site was not significant, whereas the side variation in the reference site was significant. However, the DA in the reference site was lower than FA4a (Palmer and Strobeck, 2003), indicating negligible degree of DA and enabling further FA analysis. Two-way ANOVA also indicated that the individual variation was significant in both sites, suggesting size/shape variation in these populations. The interaction of side and individual, or an indicative of fluctuating asymmetry, was present in these populations.

Table 5.7 Two-way ANOVA of male radioulna weight

	Reference site (N=83)		Contaminated site (N=68)	
	Mean square	p-value	Mean square	p-value
Side variation	4.10×10^{-7}	< 0.05*	8.26×10^{-8}	0.26
Individual variation	7.59×10^{-6}	< 0.05*	2.60×10^{-6}	< 0.05*
Interaction (sid. x ind.)	7.89×10^{-8}	< 0.05*	1.29×10^{-7}	< 0.05*
residual	3.96×10^{-8}	-	6.53×10^{-8}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Male Humerous Weight

Two–way ANOVA showed a significant side variation in the contaminated site population, but not the reference site population. However the side variation did not confound the FA analysis since the DA in the contaminated site was lower than the FA4a (Palmer and Strobeck, 2003). The individual variation as well as the interaction of side and individual were significant in both populations, suggesting the presences of size/shape variation as well as the fluctuating asymmetry.

Table 5.8 Two-way ANOVA of male humerous weight

	Reference site (N=80)		Contaminated site (N=68)	
	Mean square	p-value	Mean square	p-value
Side variation	1.10×10^{-9}	0.85	9.43×10^{-7}	< 0.05*
Individual variation	1.87×10^{-5}	< 0.05*	1.11×10^{-5}	< 0.05*
Interaction (sid. x ind.)	9.93×10^{-8}	< 0.05*	3.26×10^{-7}	< 0.05*
residual	3.42×10^{-8}	-	8.24×10^{-8}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Male Tibiofibula Weight

The results of two–way ANOVA showed a significant side variation in only the reference site population. But the DA from this population was still lower than FA4a, suggesting no interference of the DA to further FA analysis (Palmer and Strobeck, 2003). Again, the individual variation and the interaction between side and variation were significant in both populations, suggesting presences of size/shape variation fluctuating asymmetry in these populations.

Table 5.9 Two-way ANOVA of male tibiofibula weight

	Reference site (N=89)		Contaminated site (N=66)	
	Mean square	p-value	Mean square	p-value
Side variation	5.04×10^{-7}	< 0.05*	3.50×10^{-8}	0.48
Individual variation	1.46×10^{-4}	< 0.05*	6.07×10^{-5}	< 0.05*
Interaction (sid. x ind.)	2.22×10^{-7}	< 0.05*	1.16×10^{-6}	< 0.05*
residual	5.40×10^{-8}	-	7.20×10^{-8}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Male Femur Weight

Two–way ANOVA of male femur weight revealed that the side varation, the individual variation and the interaction of side and individual were all significant in these two populations. However, the side variation was minute since the DAs was lower than FA4a at both sites (Palmer and Strobeck, 2003). Therefore, it could be concluded from this two-way ANOVA that size/shape variation and fluctuating asymmetry were present in these populations.

Table 5.10 Two-way ANOVA of male femur weight

	Reference site (N=86)		Contaminated site (N=69)	
	Mean square	p-value	Mean square	p-value
Side variation	1.12×10^{-7}	< 0.05*	5.57×10^{-6}	< 0.05*
Individual variation	8.26×10^{-5}	< 0.05*	2.84×10^{-5}	< 0.05*
Interaction (sid. x ind.)	1.59×10^{-7}	< 0.05*	6.29×10^{-7}	< 0.05*
residual	4.20×10^{-7}	-	1.13×10^{-8}	-

Remark: An asterisk (*) indicates significant variation or interaction.

3.2 Comparison of FA in Male Bone Weight between Sites

After two-way ANOVA of each individual bone weight, the FA10 of each bone weight in each population was calculated according to Palmer (1994). These FA10 values were compared between sites based on the Fisher-Snedecor distribution. Similar to the results of male bone length, it was found that FA values of humerous, tibiofibula and femur weight in the contaminated site were significantly higher than those in the reference site (Table 5.11).

Table 5.11 Fluctuating asymmetry of bone weight of male rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

male-bone	Reference site		Contaminated site		F	p-value
	Df	FA	Df	FA		
radioulna	17.89	1.97×10^{-8} (N=83)	13.77	3.22×10^{-8} (N=68)	1.63	0.16
humerous	31.68	3.26×10^{-8} (N=80)	35.71	1.21×10^{-7}* (N=68)	3.71	< 0.05
tibiofibula	49.20	8.42×10^{-8} (N=89)	55.38	5.48×10^{-7}* (N=66)	6.50	< 0.05
femur	44.08	5.81×10^{-8} (N=86)	45.04	2.58×10^{-7}* (N=69)	4.44	< 0.05

Remark: An asterisk (*) indicates significant difference from the reference site.

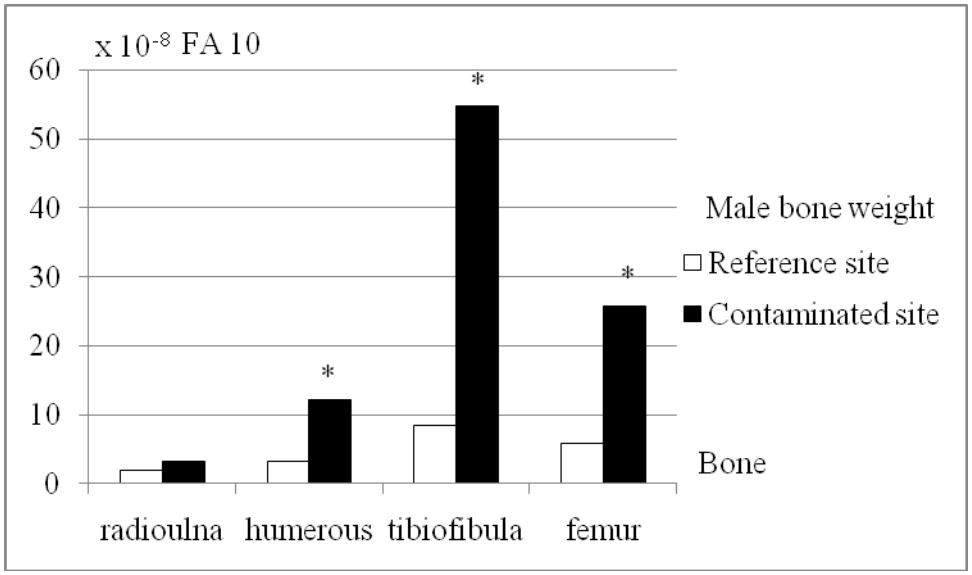


Figure 5.5 Fluctuating asymmetry of bone weight of male rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remark: An asterisk (*) indicates significant difference from the reference site.

4. Fluctuating Asymmetry of Female Bone Length

4.1 Two-way ANOVA to Test for FA Assumption

Female Radioulna Length

Similar to the male radioulna length, the results of two–way ANOVA on female radioulna length showed no significant side variation in both populations. The individual variation and the interaction of side and individual, however, were significant at both sites, indicating presences of size/shape variationa and the fluctuating asymmetry in both populations.

Table 5.12 Two-way ANOVA of female radioulna length

	Reference site (N=68)		Contaminated site (N=83)	
	Mean square	p-value	Mean square	p-value
Side variation	2.01×10^{-2}	0.16	1.41×10^{-2}	0.29
Individual variation	4.69	< 0.05*	2.76	< 0.05*
Interaction (sid. x ind.)	3.93×10^{-2}	< 0.05*	3.61×10^{-2}	< 0.05*
residual	1.05×10^{-2}	-	1.30×10^{-2}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Female Humerous Length

Comparable to the male humerous length, two–way ANOVA of female humerous length also showed significant variations in side, individual as well as interaction between side and individual in these two populations. Further comparison of the directional asymmetry and FA4a showed that the FA assumption was still met since the DA was lower than the FA4a (Palmer and Strobeck, 2003). The results of two-way ANOVA indicate the presence of size/shape variation as well as fluctuating asymmetry in these populations.

Table 5.13 Two-way ANOVA of female humerous length

	Reference site (N=69)		Contaminated site (N=85)	
	Mean square	p-value	Mean square	p-value
Side variation	7.16×10^{-2}	< 0.05*	2.07×10^{-2}	< 0.05*
Individual variation	9.34	< 0.05*	4.72	< 0.05*
Interaction (sid. x ind.)	6.90×10^{-3}	< 0.05*	1.69×10^{-2}	< 0.05*
residual	4.00×10^{-3}	-	5.00×10^{-3}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Female Tibiofibula Length

Significant variations in side, individual and interaction between side and individual in these two populations were revealed by the two–way ANOVA. However, the DA was lower than FA4a in both populations, suggesting possible FA analysis in these populations (Palmer and Strobeck, 2003). The significant individual variation and interaction between side and individual indicated that size/shape variation and fluctuating asymmetry were present in these two populations.

Table 5.14 Two-way ANOVA of female tibiofibula length

	Reference site (N=68)		Contaminated site (N=86)	
	Mean square	p-value	Mean square	p-value
Side variation	6.20×10^{-2}	< 0.05*	8.90×10^{-2}	< 0.05*
Individual variation	38.34	< 0.05*	35.78	< 0.05*
Interaction (sid. x ind.)	2.40×10^{-2}	< 0.05*	6.60×10^{-2}	< 0.05*
residual	7.00×10^{-3}	-	9.00×10^{-3}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Female Femur Length

Similar to the female humerous and tibiofibula lengths, two-way ANOVA showed that the side variation, the individual variation and the interaction of side and individual were all significant in both reference and contaminated site populations. In order to determine the FA, an assumption that the DA must be lower than FA4a (Palmer and Strobeck, 2003) was tested and successfully met. The significant individual variation indicated the presence of size/shape variation, while the significant interaction of side and individual indicated the presence of fluctuating asymmetry in these two populations.

Table 5.15 Two-way ANOVA of female femur length

	Reference site (N=66)		Contaminated site (N=87)	
	Mean square	p-value	Mean square	p-value
Side variation	3.02×10^{-2}	< 0.05*	6.42×10^{-2}	< 0.05*
Individual variation	24.53	< 0.05*	18.40	< 0.05*
Interaction (sid. x ind.)	8.80×10^{-3}	< 0.05*	1.37×10^{-2}	< 0.05*
residual	3.90×10^{-3}	-	6.60×10^{-3}	-

Remark: An asterisk (*) indicates significant variation or interaction.

4.2 Comparison of FA in Female Bone Length between Sites

After two-way ANOVA for each bone in each population was completed, FA10 were calculated according to Palmer (1994). Comparisons of the site-related difference in FA10 based on the Fisher-Snedecor distribution were performed. The results showed that fluctuating asymmetry of humerous and tibiofibula length in the contaminated site was significantly higher than those in the reference site (Table 5.16).

Table 5.16 Fluctuating asymmetry of bone length of female rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

female-bone	Reference site		Contaminated site		F	p-value
	Df	FA	Df	FA		
radioulna	33.26	0.0144 (N=68)	31.32	0.0115 (N=83)	0.80	0.73
humerous	9.40	0.0015 (N=69)	39.79	0.0060* (N=85)	4.00	< 0.05
tibiofibula	32.19	0.0088 (N=68)	61.71	0.0283* (N=86)	3.22	< 0.05
femur	17.13	0.0024 (N=66)	19.96	0.0035 (N=87)	1.45	0.21

Remark: An asterisk (*) indicates significant difference from the reference site.

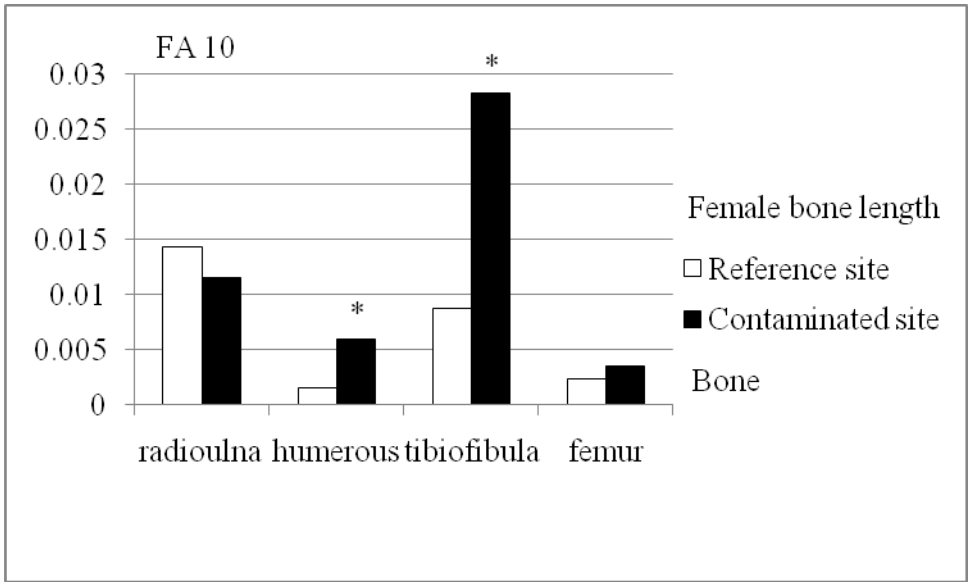


Figure 5.6 Fluctuating asymmetry of bone length of female rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

Remark: An asterisk (*) indicates significant difference from the reference site.

5. Fluctuating Asymmetry of Female Bone Weight

5.1 Two-way ANOVA to Test for FA Assumption

Female Radioulna Weight

Two–way ANOVA of female radioulna bone weight of frogs from both sites showed significant variations in side, individual as well as interaction between side and individual. In order to determine the FA, the DA was proven to be lower than FA4a (Palmer and Strobeck, 2003). As a result, it could be concluded from the two-way ANOVA that the size/shape variation and the fluctuating asymmetry were present in these two populations.

Table 5.17 Two-way ANOVA of female radioulna weight

	Reference site (N=68)		Contaminated site (N=82)	
	Mean square	p-value	Mean square	p-value
Side variation	5.03×10^{-7}	< 0.05*	6.57×10^{-7}	< 0.05*
Individual variation	2.61×10^{-5}	< 0.05*	1.91×10^{-5}	< 0.05*
Interaction (sid. x ind.)	2.15×10^{-7}	< 0.05*	2.99×10^{-7}	< 0.05*
residual	3.56×10^{-8}	-	1.10×10^{-7}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Female Humerous Weight

The results of two–way ANOVA showed the significant side variation in only the contaminated site population, but not the reference site population. However, the side variation in the contaminated site was examined to verify that the DA in this population was lower than FA4a (Palmer and Strobeck, 2003). Two-way ANOVA also indicated the significant individual variation as well as the interaction between side and individual, suggesting the presences of size/shape variation and fluctuating asymmetry (FA) in these populations.

Table 5.18 Two-way ANOVA of female humerous weight

	Reference site (N=70)		Contaminated site (N=85)	
	Mean square	p-value	Mean square	p-value
Side variation	1.10×10^{-7}	0.85	9.43×10^{-9}	< 0.05*
Individual variation	1.87×10^{-5}	< 0.05*	1.11×10^{-5}	< 0.05*
Interaction (sid. x ind.)	9.93×10^{-7}	< 0.05*	3.26×10^{-8}	< 0.05*
residual	3.42×10^{-8}	-	8.24×10^{-8}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Female Tibiofibula Weight

Unlike other female bone weight, two–way ANOVA of the female tibiofibula weight did not show any significant side variation in any populations, suggesting no directional asymmetry was present. However, the individual variation and interaction of side and individual were significant and indicated size/shape variation and fluctuating asymmetry in these populations.

Table 5.19 Two-way ANOVA of female tibiofibula weight

	Reference site (N=68)		Contaminated site (N=86)	
	Mean square	p-value	Mean square	p-value
Side variation	3.70×10^{-7}	0.37	3.20×10^{-7}	0.14
Individual variation	4.34×10^{-4}	< 0.05*	6.29×10^{-4}	< 0.05*
Interaction (sid. x ind.)	1.11×10^{-6}	< 0.05*	3.98×10^{-6}	< 0.05*
residual	4.70×10^{-7}	-	1.50×10^{-7}	-

Remark: An asterisk (*) indicates significant variation or interaction.

Female Femur Weight

The results of two–way ANOVA showed the significant side variation in the reference site population, but not in the contaminated site populations. In order to account for this difference, DA of reference site was compared with FA4a and found to be lower than FA4a, suggesting that the DA was negligible (Palmer and Strobeck, 2003). The individual variation and the interaction between side and individual were similarly significant at both sites, indicating presences of size/shape variation and fluctuating asymmetry in these populations.

Table 5.20 Two-way ANOVA of female femur weight

	Reference site (N=67)		Contaminated site (N=87)	
	Mean square	p-value	Mean square	p-value
Side variation	2.08×10^{-6}	< 0.05*	5.47×10^{-7}	0.31
Individual variation	2.42×10^{-4}	< 0.05*	2.20×10^{-4}	< 0.05*
Interaction (sid. x ind.)	1.15×10^{-7}	< 0.05*	6.75×10^{-6}	< 0.05*
residual	2.00×10^{-8}	-	5.20×10^{-7}	-

Remark: An asterisk (*) indicates significant variation or interaction.

5.2 Comparison of FA in Female Bone Weight between Sites

After two-way ANOVA for bone weight was performed, it could be concluded that FA was present in every limb bone at both populations. Therefore, FA of each bone in each population was calculated using FA10 formula (Palmer, 1994). Comparisons of FA10 between the reference site and contaminated site populations based on the Fisher-Snedecor distribution were performed. Similar to female bone length, the results showed that FA of humerous and tibiofibula were significantly different between site, with the higher value in the contaminated site (Table 5.21).

Table 5.21 Fluctuating asymmetry of bone weight of female rice frog *Fejervarya limnocharis* from reference and contaminated paddy fields at Nan Province, Thailand

female-bone	Reference site		Contaminated site		F	p-value
	Df	FA	Df	FA		
radioulna	46.08	9.01×10^{-8} (N=68)	29.75	9.44×10^{-8} (N=82)	1.05	0.43
humerous	31.68	3.26×10^{-8} (N=70)	35.71	1.21×10^{-7}* (N=85)	3.71	< 0.05
tibiofibula	79.66	3.21×10^{-7} (N=68)	20.18	1.91×10^{-6}* (N=86)	5.95	< 0.05
femur	57.85	4.76×10^{-7} (N=67)	57.85	3.11×10^{-7} (N=87)	0.65	0.06

Remark: An asterisk (*) indicates significant difference from the reference site.

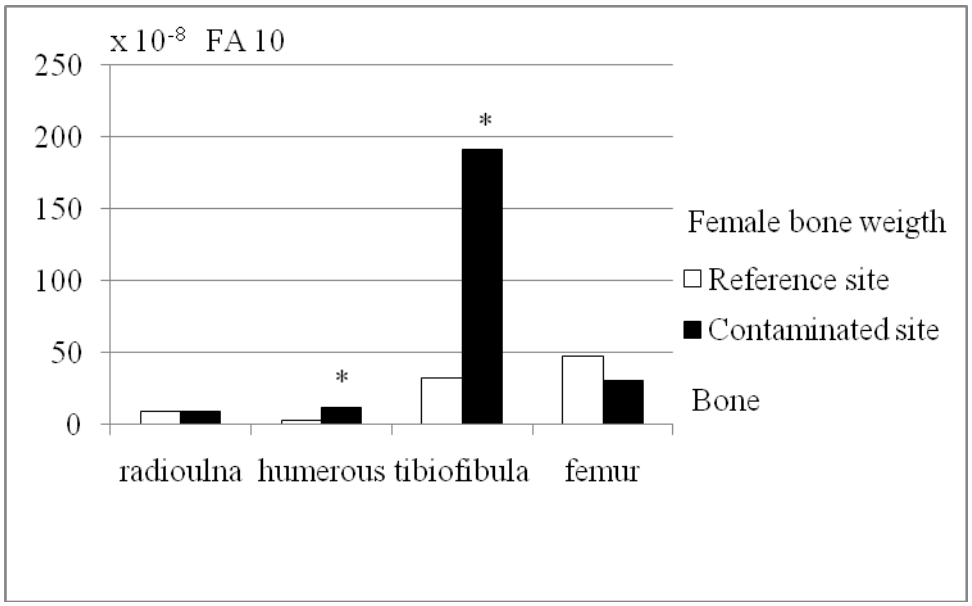


Figure 5.7 Fluctuating asymmetry of female bone weigth

Remark: An asterisk (*) indicates significant difference from the reference site.

Discussion

The result of skeletochronology showed that only small number of specimens (<10%) were present with LAGs. The absence of LAG in majority of frogs may be resulted from the arrested growth period were not clear in this frog, possibly due to climatic conditions. The climograph (Chapter III) shows that precipitation was available even in dry period, while the low temperature was brief in the dry season. Monthly field observation confirmed that frogs in these areas tended to be active in every sampling month. The absence of LAG in majority of the rice frog populations was not unexpected since previous study by Othman (2009) also indicated that LAG is not consistently present in *F. limnocharis* caught from Tak Province of Thailand. Therefore, the use of skeletochronology for age determination in this frog species in this area is not possible. Population parameters based on age of frogs were thus omitted from this study.

In fluctuating asymmetry analysis, the result showed FA was present in every appendage bones including forelimb (radioulna and humerous) and hindlimb (tibioulna and femur) of the rice frog living in both reference and contaminated sites. Comparison between sites revealed interesting results that FA values in the contaminated site population were significantly higher than those in the reference site. Since the higher fluctuating asymmetry was a result of a decrease in developmental stability and/or an increase in developmental instability (Palmer and Strobeck, 1986; 2003). It is possible that environmental stress in contaminated site may influence on developmental process of the rice frog leading to an increase in developmental instability in the frog. Since these two study sites were exposed to similar climatic condition as agricultural activities, with a major difference in herbicide utilization. Also, evidence of atrazine residue in water and significantly higher tissue residue of atrazine and paraquat were found at the contaminated site. It is highly possible that the herbicide utilization could be responsible for the increased environmental stress at the contaminated site.

Since atrazine herbicide is a well known endocrine disrupting chemical (EDC) to amphibians, it could play roles in influencing developmental process of *F. limnocharis*. In addition, glyphosate and paraquat are also toxic to amphibians.

Combination of these herbicides mixture may play a key role to increase in environmental stress in the contaminated site.

The result of FA analysis in this study was similar to previous reports on the association of environmental stressor an increase in FA of skeletal trait of frog (Soderman et al., 2007 and Helle et al., 2011). In other vertebrates, Utayopas (2001) reported that FA of fish living in polluted water in Thailand was significantly increased compared to the fish in unpolluted water. These results suggest that the FA, or asymmetry of morphological traits, is a useful tool in monitoring the influence of environmental stress on vertebrate populations.

Based on FA comparison between sites, it is of interest to note that the radioulna weight and length were not significantly different between sites, indicating lesser amount of interference from the environmental stress. This could be due to differences in time and process of appendage bone development. The development of appendage bone is a continuous process from tadpole to complete metamorphosis and after metamorphosis (Duellman and Trueb, 1994). In frog limb developmental chronology, the proximal bones (femur and humerous) develop earlier than the distal bones (tibiofibula and radioulna; Gillbert, 2010). In addition, the hindlimb was known to develop earlier than the forelimb (Duellman and Trueb, 1994). As a result, radioulna or the distal bone of the forelimb is thus developed later than the remaining appendage bones. This results in reduced time of exposure to environmental stressor during development and leading to smaller difference in fluctuating asymmetry.

It is fortunate for frogs from the contaminated site to show lesser degree of FA in radioulna bone in both male and female frogs since the radioulna is an important morphological trait for reproductive process. However, the significantly higher FA values in humerous bone of both sexes may reduce the reproductive fitness of frogs at the contaminated site in the future since the male frog must use forelimb (humerous and radioulna) to clamp on the female frog during amplexus (Soderman et al., 2007). The higher fluctuating asymmetry in this trait may impact on intrasexual selection of male frog in the contaminated site to favor the male frog with a stronger and more symmetric arm. In previous studies, the deviation from symmetry of the traits that directly influence certain performance was associated with fitness difference. It was previously reported that female barn swallows (Moller, 1992) and female

scorpionflies (Thornhill, 1991) preferred to mate with male showing more symmetrical traits.

The increase in FA on hindlimb (tibiofibula and femur) in frog from the contaminated site may greatly influence on the locomotion of frog since the hindlimb is one of an important traits for saltation, or jumping, mechanism in frog (Farish et al., 1998). Since the saltatorial locomotion is regarded as an excellent mechanisms for predator escaping in frogs (Duellman and Trueb, 1994), the higher FA in hindlimb could reduce jumping capability of frog in the contaminating site, especially when the frog was to jump to escape from the predator.

It is of interest to note that site-related difference in FA of femur was found only in male frog. The diferent result of FA between male and female frogs may be resulted from the difference in endogenous sex homones between sexes, as well as difference in level of exogenous hormone mimics between sites. Gillbert (2010) reviewed that estrogen receptors were found in cells that control production of growth hormone which regulate limb bone ossification. In female frog, endogenous estrogen is high enough to prevent interference from exogenous hormone mimics. However, in the male frog, endogenous estrogen is low making it susceptible to interference from exogenous hormone mimics. Atrazine residue was found in water of the contaminated paddy fields and its accumulation in frog tissue was evidenced (Chapter III). This endocrine disrupting chemicals could exert its estrogenic effect directly or indirectly on bone development, resulting in the higher FA of femur bone of the male frogs in the contaminated site.

Conclusion

Utilization of herbicide mixture (atrazine, glyphosate and paraquat) in agricultural activities may play the key role as environmental stressor that adversely affects population of the rice frog living in this area. Using skeletal traits and fluctuating asymmetry analysis as tools for monitoring population health, it was found that fluctuating asymmetry of frog in the contaminated was significantly higher than those from the reference site. This site-related difference suggests that herbicide utilization could affect the non-target organisms at the population level. Also, the use

of FA analysis on skeletal traits to examine the environmental stress is proven to be a useful tool for monitoring the populated rice frog.

CHAPTER VI

GENERAL CONCLUSION AND RECOMMENDATION

In this study, potential influence of herbicide utilization on the populated rice frog living in paddy fields was examined. 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. Commonly used herbicides in Nan Province include atrazine, glyphosate and paraquat. The rice frog *Fejervarya limnocharis* was used as a sentinel species to monitor the influence of herbicides on surrogate vertebrate at the level of tissue, morphology and population.

A reference and a potentially contaminated paddy fields were located in Wiang Sa District, Nan Province. The reference site (Lai-Nan Sub-District) located about 7.2 km north of the contaminated site is an organic paddy field with no history of herbicide utilization for more than 10 years. Whereas, the contaminated site (San Sub-District) is a paddy field with an intensive and prolonged herbicide utilization. Apart from 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.

In order to determine the influence of herbicides on morphology and population of rice frog, sample of the rice frog was collected on monthly basis for one year from these two study sites and subjected to examination using multiple parameters. Results of this study can be divided into three parts corresponding to different levels of biological organization, from tissue to population. The summay of the results are shown in Table 6.1

In the first part of this study, herbicide (atrazine, glyphosate and paraquat) contamination in environment was screened, and their contamination level in frog tissue was critically analyzed and compared between sites. The result of environmental contaminantion screening show that detectable amount of atrazine (0.15 mg/L) can be found in agricultural area water, while the level of glyphosate and paraquat were lower than the limit of detection of the screening test. The results of herbicide residue analysis in frog tissue showed that these three herbicides (atrazine, glyphosate and paraquat) were found in frog tissue at both site, with the significantly

higher levels of paraquat in male frog from the contaminated site during late wet period. These indicated that an intensively herbicide utilization in paddy fields of Nan Province could lead to herbicides contamination in environment as well as in non-target organism living in the affected area. The results also showed that paraquat residue in every frog samples were much higher than the maximum residue limit in food, especially in late dry period (January-March), making it unfit for human consumption. Therefore, it is importance to create public awareness that consumption of the rice frogs caught from agricultural areas should be avoided.

To examine the influence of herbicides on morphology of the rice frog, the second part of this study was carried out. Frogs were examined for condition factor as well as gravimetric and morphometric parameters and compared between two sites. It was found that the condition factor, an indicative of overall health, was decreased in frogs living in the contaminated site. In addition, the hepatosomatic index comparison showed that the frog from the contaminated site had an enlarge liver, indicating potential exposure to xenobiotics. While the female gonadosomatic index comparison showed that stimulated ovarian growth was found in the contaminated site even in the dry period where egg laying is not possible. These results suggested that the intensively use the herbicides could influence on morphology of the rice frog.

The third part of this study was carried out to determine the influence of herbicides on population of the rice frog since the herbicide contamination could be regarded as an environmental stressor that adversely affects population of the rice frog. Application of skeletochronological technique for age determination of the rice frog was unsuccessful since line of arrested growth (LAG) is not consistently presence in the cross-section of phalange bone. Therefore, population parameters based on age of the frogs could not be evaluated. The fluctuating asymmetry (FA) or the deviation from perfect symmetry of the rice frog morphological traits was used as an indicator of increased environmental stressor in the affected area. FA on appendage bone (radioulna, humerous, tibiofibula and femur) weight and length was analyzed and compared between sites. It was found that frogs living the potentially contaminated site showed significantly higher level of FA compared to those of the reference site, indicating a higher environmental stress in the herbicide utilizationa

area. The use of FA analysis to evaluate the environmental stress is suggested as a useful tool for monitoring population of the rice frog.

Overall, the results showed that the herbicide utilization could influence on morphology and population of the rice frog living in paddy fields. Additional research on health of the rice frog, especially histopathology and immunology, should be continued in order to provide link between contamination and health status of the surrogate vertebrates. These results could be used as an early warning of environmental health hazards for other vertebrates living near the herbicide utilization area, including human.

Table 6.1 Summary of the site-related difference in multiple parameters used for monitoring influence of herbicide on morphology and population of the rice frog *Fejervarya limnocharis* in paddy fields of Nan Province

Parameters	Values		Remarks
	Reference site	Contaminated site	
Contaminant analysis			
Environmental sample			
Atrazine	N/A	0.15 mg/L	In water of January 2010
Glyphosate	N/D	N/D	
Paraquat	N/D	N/D	
Biological sample (frog tissue)			
Atrazine	5.05±0.67 µg/kg	8.70±1.44 µg/kg	No significant difference between sites
Glyphosate	6.19±1.31 µg/kg	7.90±1.68 µg/kg	No significant difference between sites
Paraquat	56.6 ±4.50 µg/kg	88.80±13.07 µg/kg	No significant difference between sites

Remark:

N/D = The contamination levels were lower than the limit of detection

Parameters	Values		Remarks
	Reference site	Contaminated site	
Morphometric & gravimetric analysis			
Condition factor	104.54 ± 0.70	100.11 ± 0.73	Significant difference between sites
Hepatosomatic index (male)	1.78 ± 0.04	2.05 ± 0.07	Significant difference between sites
Hepatosomatic index (female)	2.05 ± 0.05	2.35 ± 0.08	Significant difference between sites
Renosomatic index (male)	0.40 ± 0.00	0.39 ± 0.00	No significant difference between sites
Renosomatic index (female)	0.41 ± 0.01	0.41 ± 0.01	No significant difference between sites
Gonadosomatic index (testis)	0.23 ± 0.00	0.25 ± 0.00	No significant difference between sites
Gonadosomatic index (ovary)	6.13 ± 0.75	9.93 ± 0.76	Significant difference between sites

Parameters		Values		Remarks
		Reference site	Contaminated site	
Fluctuating asymmetry				
Radioulna (male)	weight	1.97×10^{-8}	3.22×10^{-8}	No significant difference between sites
	length	0.0125	0.0157	No significant difference between sites
Radioulna (female)	weight	9.01×10^{-8}	9.44×10^{-8}	No significant difference between sites
	length	0.0144	0.0115	No significant difference between sites
Humerous (male)	weight	3.26×10^{-8}	1.21×10^{-7}	Significant difference between sites
	length	0.0040	0.0164	Significant difference between sites
Humerous (female)	weight	3.26×10^{-8}	1.21×10^{-7}	Significant difference between sites
	length	0.0015	0.0060	Significant difference between sites
Tibiofibula (male)	weight	2.58×10^{-7}	5.48×10^{-7}	Significant difference between sites
	length	0.0039	0.0143	Significant difference between sites

Parameters		Values		Remarks
		Reference site	Contaminated site	
Tibiofibula (female)	weight	3.21×10^{-7}	1.91×10^{-6}	Significant difference between sites
	length	0.0088	0.0283	Significant difference between sites
Femur (male)	weight	5.81×10^{-8}	8.42×10^{-7}	Significant difference between sites
	length	0.0023	0.0130	Significant difference between sites
Femur (female)	weight	4.76×10^{-7}	3.11×10^{-7}	No significant difference between sites
	length	0.0024	0.0035	No significant difference between sites

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APPENDICE

APPENDIX A

Sampling Data of the Rice Frog

CODE	SEX	MONTH	PERIOD
R0701	Male	July 2010	Late Wet
R0702	Male	July 2010	Late Wet
R0703	Male	July 2010	Late Wet
R0704	Male	July 2010	Late Wet
R0705	Male	July 2010	Late Wet
R0706	Female	July 2010	Late Wet
R0707	Female	July 2010	Late Wet
R0708	Female	July 2010	Late Wet
R0709	Female	July 2010	Late Wet
R0710	Female	July 2010	Late Wet
R0711	Male	July 2010	Late Wet
R0712	Male	July 2010	Late Wet
R0713	Male	July 2010	Late Wet
R0714	Male	July 2010	Late Wet
R0715	Male	July 2010	Late Wet
R0716	Male	July 2010	Late Wet
R0717	Female	July 2010	Late Wet
R0718	Male	July 2010	Late Wet
R0719	Male	July 2010	Late Wet
R0720	Male	July 2010	Late Wet
R0721	Female	July 2010	Late Wet
R0722	Female	July 2010	Late Wet
R0723	Female	July 2010	Late Wet
R0724	Male	July 2010	Late Wet
R0725	Male	July 2010	Late Wet
R0726	Male	July 2010	Late Wet
R0727	Male	July 2010	Late Wet
R0728	Male	July 2010	Late Wet

CODE	SEX	MONTH	PERIOD
R0729	Male	July 2010	Late Wet
R0730	Male	July 2010	Late Wet
R0731	Male	July 2010	Late Wet
R0732	Male	July 2010	Late Wet
R0733	Male	July 2010	Late Wet
R0734	Male	July 2010	Late Wet
R0735	Male	July 2010	Late Wet
R0736	Male	July 2010	Late Wet
R0737	Female	July 2010	Late Wet
R0738	Female	July 2010	Late Wet
R0739	Female	July 2010	Late Wet
R0740	Female	July 2010	Late Wet
R0741	Female	July 2010	Late Wet
R0801	Juvinile	August 2010	Late Wet
R0802	Juvinile	August 2010	Late Wet
R0803	Juvinile	August 2010	Late Wet
R0804	Juvinile	August 2010	Late Wet
R0805	Juvinile	August 2010	Late Wet
R0806	Juvinile	August 2010	Late Wet
R0807	Juvinile	August 2010	Late Wet
R0808	Juvinile	August 2010	Late Wet
R0809	Juvinile	August 2010	Late Wet
R0810	Juvinile	August 2010	Late Wet
R0812	Juvinile	August 2010	Late Wet
R0816	Juvinile	August 2010	Late Wet
R0817	Juvinile	August 2010	Late Wet
R0818	Juvinile	August 2010	Late Wet
R0819	Juvinile	August 2010	Late Wet

CODE	SEX	MONTH	PERIOD
R0820	Juvinile	August 2010	Late Wet
R0821	Juvinile	August 2010	Late Wet
R0822	Male	August 2010	Late Wet
R0823	Male	August 2010	Late Wet
R0824	Male	August 2010	Late Wet
R0825	Juvinile	August 2010	Late Wet
R0826	Male	August 2010	Late Wet
R0827	Female	August 2010	Late Wet
R0828	Male	August 2010	Late Wet
R0829	Male	August 2010	Late Wet
R0830	Male	August 2010	Late Wet
R0831	Juvinile	August 2010	Late Wet
R0832	Juvinile	August 2010	Late Wet
R0833	Male	August 2010	Late Wet
R0834	Male	August 2010	Late Wet
R0901	Female	September 2010	Late Wet
R0902	Female	September 2010	Late Wet
R0903	Female	September 2010	Late Wet
R0904	Female	September 2010	Late Wet
R0905	Male	September 2010	Late Wet
R0906	Male	September 2010	Late Wet
R0907	Female	September 2010	Late Wet
R0908	Female	September 2010	Late Wet
R0909	Male	September 2010	Late Wet
R0910	Male	September 2010	Late Wet
R0911	Male	September 2010	Late Wet
R0912	Female	September 2010	Late Wet
R0913	Male	September 2010	Late Wet

CODE	SEX	MONTH	PERIOD
R0914	Female	September 2010	Late Wet
R0915	Female	September 2010	Late Wet
R0916	Male	September 2010	Late Wet
R0917	Male	September 2010	Late Wet
R0918	Male	September 2010	Late Wet
R1001	Female	October 2010	Early Dry
R1002	Female	October 2010	Early Dry
R1003	Female	October 2010	Early Dry
R1004	Female	October 2010	Early Dry
R1005	Female	October 2010	Early Dry
R1006	Male	October 2010	Early Dry
R1007	Male	October 2010	Early Dry
R1008	Male	October 2010	Early Dry
R1009	Male	October 2010	Early Dry
R1010	Male	October 2010	Early Dry
R1011	Female	October 2010	Early Dry
R1012	Female	October 2010	Early Dry
R1013	Male	October 2010	Early Dry
R1014	Female	October 2010	Early Dry
R1015	Female	October 2010	Early Dry
R1016	Female	October 2010	Early Dry
R1017	Juvinile	October 2010	Early Dry
R1018	Female	October 2010	Early Dry
R1019	Juvinile	October 2010	Early Dry
R1020	Female	October 2010	Early Dry
R1021	Juvinile	October 2010	Early Dry
R1022	Juvinile	October 2010	Early Dry
R1023	Juvinile	October 2010	Early Dry

CODE	SEX	MONTH	PERIOD
R1024	Juvinile	October 2010	Early Dry
R1025	Juvinile	October 2010	Early Dry
R1026	Juvinile	October 2010	Early Dry
R1027	Juvinile	October 2010	Early Dry
R1028	Juvinile	October 2010	Early Dry
R1029	Juvinile	October 2010	Early Dry
R1030	Juvinile	October 2010	Early Dry
R1031	Juvinile	October 2010	Early Dry
R1032	Juvinile	October 2010	Early Dry
R1033	Juvinile	October 2010	Early Dry
R1034	Juvinile	October 2010	Early Dry
R1035	Juvinile	October 2010	Early Dry
R1036	Juvinile	October 2010	Early Dry
R1037	Juvinile	October 2010	Early Dry
R1038	Juvinile	October 2010	Early Dry
R1039	Juvinile	October 2010	Early Dry
R1040	Juvinile	October 2010	Early Dry
R1041	Juvinile	October 2010	Early Dry
R1042	Juvinile	October 2010	Early Dry
R1043	Juvinile	October 2010	Early Dry
R1044	Juvinile	October 2010	Early Dry
R1045	Juvinile	October 2010	Early Dry
R1046	Juvinile	October 2010	Early Dry
R1047	Juvinile	October 2010	Early Dry
R1048	Juvinile	October 2010	Early Dry
R1049	Juvinile	October 2010	Early Dry
R1050	Juvinile	October 2010	Early Dry
R1051	Juvinile	October 2010	Early Dry

CODE	SEX	MONTH	PERIOD
R1052	Juvinile	October 2010	Early Dry
R1053	Juvinile	October 2010	Early Dry
R1054	Juvinile	October 2010	Early Dry
R1101	Juvinile	November 2010	Early Dry
R1102	Juvinile	November 2010	Early Dry
R1103	Juvinile	November 2010	Early Dry
R1104	Juvinile	November 2010	Early Dry
R1105	Juvinile	November 2010	Early Dry
R1106	Juvinile	November 2010	Early Dry
R1107	Juvinile	November 2010	Early Dry
R1108	Juvinile	November 2010	Early Dry
R1109	Male	November 2010	Early Dry
R1110	Female	November 2010	Early Dry
R1111	Juvinile	November 2010	Early Dry
R1112	Juvinile	November 2010	Early Dry
R1201	Female	December 2010	Early Dry
R1202	Female	December 2010	Early Dry
R1203	Female	December 2010	Early Dry
R1204	Female	December 2010	Early Dry
R1205	Female	December 2010	Early Dry
R1206	Juvinile	December 2010	Early Dry
R1207	Juvinile	December 2010	Early Dry
R1208	Juvinile	December 2010	Early Dry
R1209	Juvinile	December 2010	Early Dry
R1210	Juvinile	December 2010	Early Dry
R1211	Juvinile	December 2010	Early Dry
R0101	Male	January 2011	Late Dry
R0102	Male	January 2011	Late Dry

CODE	SEX	MONTH	PERIOD
R0103	Male	January 2011	Late Dry
R0104	Female	January 2011	Late Dry
R0105	Female	January 2011	Late Dry
R0106	Female	January 2011	Late Dry
R0107	Juvinile	January 2011	Late Dry
R0108	Juvinile	January 2011	Late Dry
R0109	Juvinile	January 2011	Late Dry
R0110	Juvinile	January 2011	Late Dry
R0111	Juvinile	January 2011	Late Dry
R0112	Juvinile	January 2011	Late Dry
R0113	Juvinile	January 2011	Late Dry
R0114	Male	January 2011	Late Dry
R0115	Male	January 2011	Late Dry
R0116	Female	January 2011	Late Dry
R0117	Male	January 2011	Late Dry
R0118	Juvinile	January 2011	Late Dry
R0119	Male	January 2011	Late Dry
R0120	Male	January 2011	Late Dry
R0121	Male	January 2011	Late Dry
R0122	Female	January 2011	Late Dry
R0123	Female	January 2011	Late Dry
R0124	Female	January 2011	Late Dry
R0201	Female	Febuary 2011	Late Dry
R0202	Female	Febuary 2011	Late Dry
R0203	Female	Febuary 2011	Late Dry
R0205	Female	Febuary 2011	Late Dry
R0206	Female	Febuary 2011	Late Dry
R0207	Male	Febuary 2011	Late Dry

CODE	SEX	MONTH	PERIOD
R0208	Male	Febuary 2011	Late Dry
R0209	Male	Febuary 2011	Late Dry
R0210	Female	Febuary 2011	Late Dry
R0211	Female	Febuary 2011	Late Dry
R0212	Female	Febuary 2011	Late Dry
R0213	Female	Febuary 2011	Late Dry
R0214	Female	Febuary 2011	Late Dry
R0215	Female	Febuary 2011	Late Dry
R0216	Male	Febuary 2011	Late Dry
R0217	Male	Febuary 2011	Late Dry
R0218	Male	Febuary 2011	Late Dry
R0219	Male	Febuary 2011	Late Dry
R0301	Male	March 2011	Late Dry
R0302	Male	March 2011	Late Dry
R0303	Male	March 2011	Late Dry
R0304	Male	March 2011	Late Dry
R0305	Male	March 2011	Late Dry
R0306	Male	March 2011	Late Dry
R0307	Female	March 2011	Late Dry
R0308	Female	March 2011	Late Dry
R0309	Female	March 2011	Late Dry
R0310	Female	March 2011	Late Dry
R0311	Male	March 2011	Late Dry
R0312	Male	March 2011	Late Dry
R0313	Male	March 2011	Late Dry
R0314	Male	March 2011	Late Dry
R0315	Male	March 2011	Late Dry
R0316	Male	March 2011	Late Dry

CODE	SEX	MONTH	PERIOD
R0317	Male	March 2011	Late Dry
R0318	Male	March 2011	Late Dry
R0319	Female	March 2011	Late Dry
R0401	Female	April 2011	Early wet
R0402	Male	April 2011	Early wet
R0403	Male	April 2011	Early wet
R0404	Female	April 2011	Early wet
R0405	Female	April 2011	Early wet
R0406	Female	April 2011	Early wet
R0407	Male	April 2011	Early wet
R0408	Male	April 2011	Early wet
R0409	Male	April 2011	Early wet
R0410	Male	April 2011	Early wet
R0411	Male	April 2011	Early wet
R0412	Male	April 2011	Early wet
R0413	Male	April 2011	Early wet
R0414	Male	April 2011	Early wet
R0415	Male	April 2011	Early wet
R0416	Male	April 2011	Early wet
R0417	Female	April 2011	Early wet
R0418	Female	April 2011	Early wet
R0419	Female	April 2011	Early wet
R0420	Female	April 2011	Early wet
R0421	Female	April 2011	Early wet
R0422	Female	April 2011	Early wet
R0501	Male	May 2011	Early wet
R0502	Male	May 2011	Early wet
R0503	Male	May 2011	Early wet

CODE	SEX	MONTH	PERIOD
R0504	Male	May 2011	Early wet
R0505	Male	May 2011	Early wet
R0506	Male	May 2011	Early wet
R0507	Male	May 2011	Early wet
R0508	Male	May 2011	Early wet
R0509	Male	May 2011	Early wet
R0510	Male	May 2011	Early wet
R0511	Male	May 2011	Early wet
R0512	Female	May 2011	Early wet
R0513	Female	May 2011	Early wet
R0514	Female	May 2011	Early wet
R0515	Female	May 2011	Early wet
R0516	Female	May 2011	Early wet
R0517	Female	May 2011	Early wet
R0518	Female	May 2011	Early wet
R0519	Female	May 2011	Early wet
R0520	Female	May 2011	Early wet
R0521	Female	May 2011	Early wet
RR0601	Female	June 2011	Early wet
RR0602	Female	June 2011	Early wet
RR0603	Female	June 2011	Early wet
RR0604	Female	June 2011	Early wet
RR0605	Female	June 2011	Early wet
RR0606	Female	June 2011	Early wet
RR0607	Juvinile	June 2011	Early wet
RR0609	Juvinile	June 2011	Early wet
RR0611	Male	June 2011	Early wet
RR0612	Male	June 2011	Early wet
RR0613	Male	June 2011	Early wet

CODE	SEX	MONTH	PERIOD
RR0614	Male	June 2011	Early wet
RR0615	Male	June 2011	Early wet
RR0617	Male	June 2011	Early wet
RR0618	Male	June 2011	Early wet
C0701	Male	July 2010	Late Wet
C0702	Male	July 2010	Late Wet
C0703	Male	July 2010	Late Wet
C0704	Male	July 2010	Late Wet
C0705	Male	July 2010	Late Wet
C0706	Female	July 2010	Late Wet
C0707	Female	July 2010	Late Wet
C0708	Female	July 2010	Late Wet
C0709	Female	July 2010	Late Wet
C0710	Female	July 2010	Late Wet
C0711	Male	July 2010	Late Wet
C0712	Male	July 2010	Late Wet
C0713	Male	July 2010	Late Wet
C0714	Male	July 2010	Late Wet
C0715	Male	July 2010	Late Wet
C0716	Female	July 2010	Late Wet
C0717	Female	July 2010	Late Wet
C0718	Male	July 2010	Late Wet
C0719	Male	July 2010	Late Wet
C0720	Male	July 2010	Late Wet
C0721	Male	July 2010	Late Wet
C0722	Male	July 2010	Late Wet
C0723	Female	July 2010	Late Wet
C0724	Female	July 2010	Late Wet

CODE	SEX	MONTH	PERIOD
C0725	Female	July 2010	Late Wet
C0726	Female	July 2010	Late Wet
C0727	Male	July 2010	Late Wet
C0728	Male	July 2010	Late Wet
C0729	Male	July 2010	Late Wet
C0730	Male	July 2010	Late Wet
C0731	Male	July 2010	Late Wet
C0732	Female	July 2010	Late Wet
C0733	Female	July 2010	Late Wet
C0734	Female	July 2010	Late Wet
C0735	Female	July 2010	Late Wet
C0736	Female	July 2010	Late Wet
C0737	Female	July 2010	Late Wet
C0801	Female	August 2010	Late Wet
C0802	Female	August 2010	Late Wet
C0803	Male	August 2010	Late Wet
C0804	Male	August 2010	Late Wet
C0805	Female	August 2010	Late Wet
C0806	Female	August 2010	Late Wet
C0807	Female	August 2010	Late Wet
C0808	Juvinile	August 2010	Late Wet
C0809	Male	August 2010	Late Wet
C0810	Juvinile	August 2010	Late Wet
C0811	Female	August 2010	Late Wet
C0812	Female	August 2010	Late Wet
C0813	Juvinile	August 2010	Late Wet
C0814	Female	August 2010	Late Wet
C0815	Male	August 2010	Late Wet

CODE	SEX	MONTH	PERIOD
C0816	Female	August 2010	Late Wet
C0817	Female	August 2010	Late Wet
C0818	Female	August 2010	Late Wet
C0819	Female	August 2010	Late Wet
C0820	Male	August 2010	Late Wet
C0821	Female	August 2010	Late Wet
C0822	Female	August 2010	Late Wet
C0901	Female	Sepetember 2010	Late Wet
C0902	Female	Sepetember 2010	Late Wet
C0903	Female	Sepetember 2010	Late Wet
C0904	Female	Sepetember 2010	Late Wet
C0905	Female	Sepetember 2010	Late Wet
C0906	Female	Sepetember 2010	Late Wet
C0907	Female	Sepetember 2010	Late Wet
C0909	Female	Sepetember 2010	Late Wet
C0910	Male	Sepetember 2010	Late Wet
C0911	Male	Sepetember 2010	Late Wet
C0912	Male	Sepetember 2010	Late Wet
C0913	Male	Sepetember 2010	Late Wet
C0914	Juvinile	Sepetember 2010	Late Wet
C0915	Juvinile	Sepetember 2010	Late Wet
C0916	Juvinile	Sepetember 2010	Late Wet
C0917	Juvinile	Sepetember 2010	Late Wet
C0918	Juvinile	Sepetember 2010	Late Wet
C0919	Juvinile	Sepetember 2010	Late Wet
C0920	Juvinile	Sepetember 2010	Late Wet
C0921	Juvinile	Sepetember 2010	Late Wet
C0922	Juvinile	Sepetember 2010	Late Wet

CODE	SEX	MONTH	PERIOD
C0923	Juvinile	Sepetember 2010	Late Wet
C0924	Juvinile	Sepetember 2010	Late Wet
C0925	Juvinile	Sepetember 2010	Late Wet
C0926	Juvinile	Sepetember 2010	Late Wet
C0927	Juvinile	Sepetember 2010	Late Wet
C0928	Juvinile	Sepetember 2010	Late Wet
C1001	Female	October 2010	Early Dry
C1002	Female	October 2010	Early Dry
C1003	Female	October 2010	Early Dry
C1004	Female	October 2010	Early Dry
C1005	Female	October 2010	Early Dry
C1006	Male	October 2010	Early Dry
C1007	Male	October 2010	Early Dry
C1008	Male	October 2010	Early Dry
C1009	Male	October 2010	Early Dry
C1010	Male	October 2010	Early Dry
C1011	Juvinile	October 2010	Early Dry
C1012	Juvinile	October 2010	Early Dry
C1013	Juvinile	October 2010	Early Dry
C1014	Juvinile	October 2010	Early Dry
C1015	Juvinile	October 2010	Early Dry
C1016	Female	October 2010	Early Dry
C1017	Juvinile	October 2010	Early Dry
C1018	Female	October 2010	Early Dry
C1019	Male	October 2010	Early Dry
C1020	Juvinile	October 2010	Early Dry
C1021	Juvinile	October 2010	Early Dry
C1022	Juvinile	October 2010	Early Dry

CODE	SEX	MONTH	PERIOD
C1023	Juvinile	October 2010	Early Dry
C1024	Juvinile	October 2010	Early Dry
C1025	Juvinile	October 2010	Early Dry
C1026	Juvinile	October 2010	Early Dry
C1027	Juvinile	October 2010	Early Dry
C1028	Juvinile	October 2010	Early Dry
C1029	Juvinile	October 2010	Early Dry
C1030	Juvinile	October 2010	Early Dry
C1031	Juvinile	October 2010	Early Dry
C1032	Juvinile	October 2010	Early Dry
C1033	Juvinile	October 2010	Early Dry
C1034	Juvinile	October 2010	Early Dry
C1035	Juvinile	October 2010	Early Dry
C1036	Juvinile	October 2010	Early Dry
C1037	Juvinile	October 2010	Early Dry
C1038	Juvinile	October 2010	Early Dry
C1039	Juvinile	October 2010	Early Dry
C1040	Juvinile	October 2010	Early Dry
C1041	Juvinile	October 2010	Early Dry
C1042	Juvinile	October 2010	Early Dry
C1043	Juvinile	October 2010	Early Dry
C1044	Juvinile	October 2010	Early Dry
C1045	Juvinile	October 2010	Early Dry
C1046	Juvinile	October 2010	Early Dry
C1047	Juvinile	October 2010	Early Dry
C1048	Juvinile	October 2010	Early Dry
C1049	Juvinile	October 2010	Early Dry
C1050	Juvinile	October 2010	Early Dry

CODE	SEX	MONTH	PERIOD
C1051	Juvinile	October 2010	Early Dry
C1052	Juvinile	October 2010	Early Dry
C1053	Juvinile	October 2010	Early Dry
C1054	Juvinile	October 2010	Early Dry
C1055	Juvinile	October 2010	Early Dry
C1056	Juvinile	October 2010	Early Dry
C1057	Juvinile	October 2010	Early Dry
C1058	Juvinile	October 2010	Early Dry
C1101	Juvinile	November 2010	Early Dry
C1102	Juvinile	November 2010	Early Dry
C1103	Juvinile	November 2010	Early Dry
C1104	Juvinile	November 2010	Early Dry
C1105	Juvinile	November 2010	Early Dry
C1106	Juvinile	November 2010	Early Dry
C1107	Juvinile	November 2010	Early Dry
C1108	Male	November 2010	Early Dry
C1109	Female	November 2010	Early Dry
C1110	Juvinile	November 2010	Early Dry
C1111	Juvinile	November 2010	Early Dry
C1112	Juvinile	November 2010	Early Dry
C1113	Juvinile	November 2010	Early Dry
C1114	Juvinile	November 2010	Early Dry
C1115	Juvinile	November 2010	Early Dry
C1116	Juvinile	November 2010	Early Dry
C1117	Juvinile	November 2010	Early Dry
C1118	Juvinile	November 2010	Early Dry
C1119	Juvinile	November 2010	Early Dry
C1201	Female	December 2010	Early Dry

CODE	SEX	MONTH	PERIOD
C1202	Female	December 2010	Early Dry
C1203	Male	December 2010	Early Dry
C1204	Female	December 2010	Early Dry
C1205	Male	December 2010	Early Dry
C1206	Juvinile	December 2010	Early Dry
C1207	Juvinile	December 2010	Early Dry
C1208	Juvinile	December 2010	Early Dry
C1209	Female	December 2010	Early Dry
C1210	Female	December 2010	Early Dry
C0101	Male	January 2011	Late Dry
C0102	Male	January 2011	Late Dry
C0103	Male	January 2011	Late Dry
C0104	Female	January 2011	Late Dry
C0105	Female	January 2011	Late Dry
C0106	Female	January 2011	Late Dry
C0107	Female	January 2011	Late Dry
C0110	Juvinile	January 2011	Late Dry
C0111	Juvinile	January 2011	Late Dry
C0112	Juvinile	January 2011	Late Dry
C0113	Female	January 2011	Late Dry
C0114	Female	January 2011	Late Dry
C0115	Female	January 2011	Late Dry
C0117	Female	January 2011	Late Dry
C0118	Female	January 2011	Late Dry
C0119	Female	January 2011	Late Dry
C0120	Male	January 2011	Late Dry
C0121	Male	January 2011	Late Dry
C0122	Male	January 2011	Late Dry

CODE	SEX	MONTH	PERIOD
C0201	Female	Febuary 2011	Late Dry
C0202	Female	Febuary 2011	Late Dry
C0203	Female	Febuary 2011	Late Dry
C0204	Female	Febuary 2011	Late Dry
C0205	Female	Febuary 2011	Late Dry
C0206	Female	Febuary 2011	Late Dry
C0207	Female	Febuary 2011	Late Dry
C0208	Female	Febuary 2011	Late Dry
C0209	Male	Febuary 2011	Late Dry
C0210	Male	Febuary 2011	Late Dry
C0211	Male	Febuary 2011	Late Dry
C0212	Male	Febuary 2011	Late Dry
C0213	Male	Febuary 2011	Late Dry
C0214	Male	Febuary 2011	Late Dry
C0215	Female	Febuary 2011	Late Dry
C0301	Male	March 2011	Late Dry
C0302	Male	March 2011	Late Dry
C0303	Male	March 2011	Late Dry
C0304	Female	March 2011	Late Dry
C0305	Female	March 2011	Late Dry
C0306	Female	March 2011	Late Dry
C0307	Female	March 2011	Late Dry
C0401	Male	April 2011	Early Wet
C0402	Male	April 2011	Early Wet
C0403	Male	April 2011	Early Wet
C0404	Female	April 2011	Early Wet
C0405	Female	April 2011	Early Wet
C0406	Female	April 2011	Early Wet

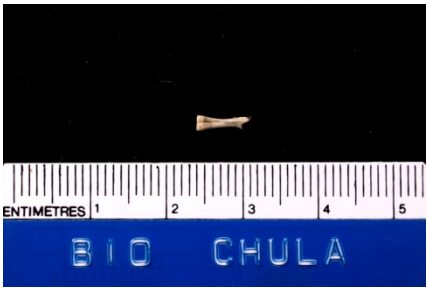
CODE	SEX	MONTH	PERIOD
C0407	Female	April 2011	Early Wet
C0408	Female	April 2011	Early Wet
C0409	Female	April 2011	Early Wet
C0410	Female	April 2011	Early Wet
C0411	Female	April 2011	Early Wet
C0412	Female	April 2011	Early Wet
C0413	Female	April 2011	Early Wet
C0414	Female	April 2011	Early Wet
C0415	Female	April 2011	Early Wet
C0416	Male	April 2011	Early Wet
C0417	Male	April 2011	Early Wet
C0418	Male	April 2011	Early Wet
C0419	Male	April 2011	Early Wet
C0420	Male	April 2011	Early Wet
C0421	Male	April 2011	Early Wet
C0422	Male	April 2011	Early Wet
C0501	Female	May 2011	Early Wet
C0502	Female	May 2011	Early Wet
C0503	Female	May 2011	Early Wet
C0504	Female	May 2011	Early Wet
C0505	Female	May 2011	Early Wet
C0506	Female	May 2011	Early Wet
C0507	Female	May 2011	Early Wet
C0508	Female	May 2011	Early Wet
C0509	Female	May 2011	Early Wet
C0510	Female	May 2011	Early Wet
C0511	Male	May 2011	Early Wet
C0512	Male	May 2011	Early Wet

CODE	SEX	MONTH	PERIOD
C0513	Male	May 2011	Early Wet
C0514	Male	May 2011	Early Wet
C0515	Male	May 2011	Early Wet
C0516	Male	May 2011	Early Wet
C0517	Male	May 2011	Early Wet
C0518	Male	May 2011	Early Wet
C0519	Male	May 2011	Early Wet
CC0601	Female	June 11	Early Wet
CC0602	Female	June 11	Early Wet
CC0603	Female	June 11	Early Wet
CC0604	Female	June 11	Early Wet
CC0605	Female	June 11	Early Wet
CC0606	Female	June 11	Early Wet
CC0607	Female	June 11	Early Wet
CC0608	Female	June 11	Early Wet
CC0609	Female	June 11	Early Wet
CC0610	Male	June 11	Early Wet
CC0611	Male	June 11	Early Wet
CC0612	Male	June 11	Early Wet
CC0613	Male	June 11	Early Wet
CC0614	Male	June 11	Early Wet
CC0615	Male	June 11	Early Wet
CC0616	Male	June 11	Early Wet
CC0617	Male	June 11	Early Wet
CC0618	Male	June 11	Early Wet
CC0619	Male	June 11	Early Wet
CC0620	Male	June 11	Early Wet
CC0621	Male	June 11	Early Wet

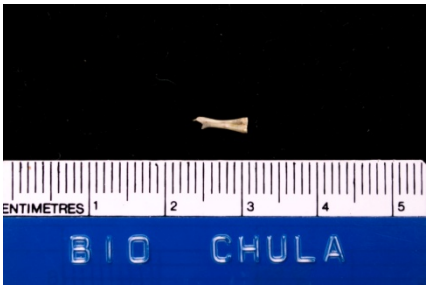
CODE	SEX	MONTH	PERIOD
CC0622	Male	June 11	Early Wet
CC0623	Male	June 11	Early Wet
CC0624	Male	June 11	Early Wet
CC0625	Male	June 11	Early Wet

APPENDIX B

Appendage Bones of the Rice Frog for Fluctuating Asymmetry Analysis

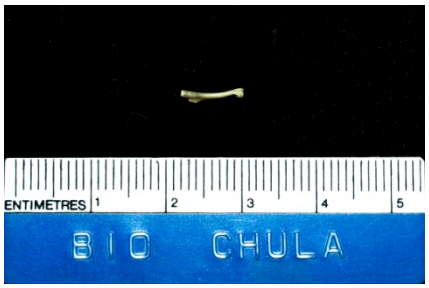


Left radioulna bone

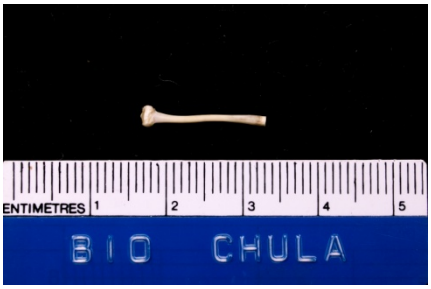


Right radioulna bone

Left humerous bone



Right humerous bone



Left tibiofibula bone

Right tibiofibula bone

Left femur bone

Right femur bone

APPENDIX C

Data of Two-way ANOVA of the Appendage Bones of the Rice Frog in Fluctuating Asymmetry Analysis

Two-way ANOVA on male radioulna weight of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.00000041	4.102×10^{-7}	10.3667	0.0015470
Individual	81	0.00061503	7.593×10^{-6}	191.8713	$< 2.2 \times 10^{-16}$
Side X individual	81	0.00000639	7.890×10^{-8}	1.9934	0.0001014
Residuals	164	0.00000649	3.960×10^{-8}		

Two-way ANOVA on male radioulna weight of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	8.3000×10^{-8}	8.266×10^{-8}	1.2640	0.2629921
Individual	63	1.6421×10^{-4}	2.6066×10^{-6}	39.8611	$< 2.2 \times 10^{-16}$
Side X individual	63	8.1720×10^{-6}	1.2972×10^{-6}	1.9838	0.0005583
Residuals	128	8.3700×10^{-6}	6.539×10^{-8}		

Two-way ANOVA on male radioulna length of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.021	0.02096	3.2172	0.07467
Individual	83	164.476	1.98164	304.1200	$< 2.2 \times 10^{-16}$
Side X individual	83	2.620	0.03156	4.8442	$< 2.2 \times 10^{-16}$
Residuals	168	1.095	0.00652		

Two-way ANOVA on male radioulna length of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.001	0.00051	0.0502	0.823
Individual	67	0.86113	0.86113	84.6410	$< 2.2 \times 10^{-16}$
Side X individual	67	0.04154	0.04154	4.0828	1.825×10^{-12}
Residuals	136	0.01017	0.01017		

Two-way ANOVA on female radioulna weight of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.00000050	5.0330×10^{-7}	14.1269	0.0002528
Individual	67	0.00175168	2.6145×10^{-5}	733.8820	$< 2.2 \times 10^{-16}$
Side X individual	67	0.00001446	2.1580×10^{-7}	6.0578	$< 2.2 \times 10^{-16}$
Residuals	136	0.00000484	3.5600×10^{-8}		

Two-way ANOVA on female radioulna weight of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	6.600×10^{-7}	6.5790×10^{-7}	5.9343	0.01593
Individual	80	1.534×10^{-3}	1.9175×10^{-5}	172.9612	$< 2.2 \times 10^{-16}$
Side X individual	80	2.898×10^{-5}	2.9980×10^{-7}	2.7040	4.321×10^{-8}
Residuals	162	1.796×10^{-5}	1.1090×10^{-7}		

Two-way ANOVA on female radioulna length of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.020	0.0201	1.9201	0.1682
Individual	64	300.730	4.6989	448.5073	$< 2.2 \times 10^{-16}$
Side X individual	64	2.515	0.0393	3.7511	8.548×10^{-11}
Residuals	130	1.362	0.0105		

Two-way ANOVA on female radioulna length of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.014	0.01418	1.0840	0.2993
Individual	82	226.847	2.76642	211.4367	$< 2.2 \times 10^{-16}$
Side X individual	82	2.962	0.03612	2.7606	1.57×10^{-8}
Residuals	166	2.172	0.01308		

Two-way ANOVA on male humerous weight of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.000×10^{0}	1.1000×10^{-9}	0.0333	0.8554
Individual	78	1.463×10^{-3}	1.8757×10^{-5}	548.8097	$< 2.2 \times 10^{-16}$
Side X individual	78	7.740×10^{-6}	9.9300×10^{-8}	2.9049	7.054×10^{-9}
Residuals	158	5.400×10^{-6}	3.4200×10^{-8}		

Two-way ANOVA on male humerous weight of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	9.400×10^{-7}	9.4330×10^{-7}	11.4445	0.0009404
Individual	66	7.364×10^{-4}	1.1158×10^{-5}	135.3663	$< 2.2 \times 10^{-16}$
Side X individual	66	2.151×10^{-5}	3.2600×10^{-7}	3.9548	7.775×10^{-12}
Residuals	134	1.104×10^{-5}	8.2400×10^{-8}		

Two-way ANOVA on male humerous length of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.157	0.15672	33.2735	5.154×10^{-8}
Individual	67	102.763	1.53378	325.6398	$< 2.2 \times 10^{-16}$
Side X individual	67	2.510	0.03747	7.9545	$< 2.2 \times 10^{-16}$
Residuals	136	0.641	0.00471		

Two-way ANOVA on male humerous length of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.030	0.0299	10.1859	0.0017
Individual	80	261.239	3.2655	1113.5069	$< 2.2 \times 10^{-16}$
Side X individual	80	0.875	0.0109	3.7292	6.248×10^{-13}
Residuals	162	0.475	0.0029		

Two-way ANOVA on female humerous weight of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.00×10^{0}	1.1000×10^{-9}	0.0333	0.8554
Individual	78	1463×10^{-3}	1.8757×10^{-5}	548.8097	$< 2.2 \times 10^{-16}$
Side X individual	78	7.740×10^{-6}	9.9300×10^{-8}	2.9049	7.054×10^{-9}
Residuals	158	5.400×10^{-6}	3.4200×10^{-8}		

Two-way ANOVA on female humerous weight of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	9.400×10^{-7}	9.4330×10^{-7}	11.4445	0.0009404
Individual	66	7.364×10^{-4}	1.1158×10^{-5}	135.3663	$< 2.2 \times 10^{-16}$
Side X individual	66	2.151×10^{-5}	3.2600×10^{-7}	3.9548	7.775×10^{-12}
Residuals	134	1.104×10^{-5}	8.2400×10^{-8}		

Two-way ANOVA on female humerous length of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.07	0.0716	17.8888	4.466×10^{-5}
Individual	62	579.47	9.3463	2335.9220	$< 2.2 \times 10^{-16}$
Side X individual	62	0.43	0.0069	1.7254	0.005111
Residuals	162	0.50	0.0040		

Two-way ANOVA on female humerous length of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.02	0.0207	4.1649	0.04283
Individual	83	392.34	4.7270	952.0177	$< 2.2 \times 10^{-16}$
Side X individual	83	1.41	0.0169	3.4130	7.818×10^{-12}
Residuals	168	0.83	0.0050		

Two-way ANOVA on male tibiofibula weight of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.0000005	5.0400×10^{-7}	9.4010	0.002507
Individual	88	0.0128599	1.4614×10^{-4}	2723.7753	$< 2.2 \times 10^{-16}$
Side X individual	88	0.0000195	2.2200×10^{-7}	4.1398	4.721×10^{-16}
Residuals	187	0.0000096	5.4000×10^{-8}		

Two-way ANOVA on male tibiofibula weight of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.0000000	3.500×10^{-8}	0.4891	0.4856
Individual	63	0.0038272	6.075×10^{-5}	845.2168	$< 2.2 \times 10^{-16}$
Side X individual	63	0.0000736	1.168×10^{-6}	16.2572	$< 2.2 \times 10^{-16}$
Residuals	128	0.0000092	7.200×10^{-8}		

Two-way ANOVA on male tibiofibula length of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.00	0.0011	0.3006	0.5842
Individual	89	1487.45	16.7129	4556.3561	$< 2.2 \times 10^{-16}$
Side X individual	89	1.02	0.0115	3.1364	4.145×10^{-11}
Residuals	180	0.66	0.0037		

Two-way ANOVA on male tibiofibula length of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.03	0.0275	5.4863	0.02069
Individual	64	601.40	9.3968	1871.7996	$< 2.2 \times 10^{-16}$
Side X individual	64	2.16	0.0337	6.7128	$< 2.2 \times 10^{-16}$
Residuals	130	0.65	0.0050		

Two-way ANOVA on female tibiofibula weight of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.0000004	0.00000037	0.7775	0.3759
Individual	66	0.0286472	0.00043405	922.8470	$< 2.2 \times 10^{-16}$
Side X individual	66	0.0000734	0.00000111	2.3631	1.335×10^{-5}
Residuals	134	0.0000630	0.00000047		

Two-way ANOVA on female tibiofibula weight of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.000000	3.20×10^{-7}	2.1404	0.1453
Individual	86	0.054094	6.29×10^{-4}	4249.4833	$< 2.2 \times 10^{-16}$
Side X individual	86	0.000342	3.98×10^{-6}	26.8888	$< 2.2 \times 10^{-16}$
Residuals	174	0.000026	1.50×10^{-7}		

Two-way ANOVA on female tibiofibula length of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.06	0.062	9.2500	0.002849
Individual	64	2453.87	38.342	5697.6373	$< 2.2 \times 10^{-16}$
Side X individual	64	1.55	0.024	3.6042	2.968×10^{-10}
Residuals	130	0.87	0.007		

Two-way ANOVA on female tibiofibula length of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.09	0.089	9.8306	0.002023
Individual	84	30005.63	35.781	3930.6597	$< 2.2 \times 10^{-16}$
Side X individual	84	5.51	0.066	7.2071	$< 2.2 \times 10^{-16}$
Residuals	170	1.55	0.009		

Two-way ANOVA on male femur weight of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.0000011	1.1280×10^{-6}	26.5997	6.831×10^{-7}
Individual	85	0.0070282	8.2685×10^{-5}	1949.5282	$< 2.2 \times 10^{-16}$
Side X individual	85	0.0000135	1.5900×10^{-7}	3.7417	1.107×10^{-13}
Residuals	172	0.0000073	4.2000×10^{-8}		

Two-way ANOVA on male femur weight of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.00000056	5.571×10^{-16}	4.928	0.02805
Individual	68	0.00193495	2.8455×10^{-16}	251.7189	$< 2.2 \times 10^{-16}$
Side X individual	68	0.00004278	6.2920×10^{-16}	5.5656	$< 2.2 \times 10^{-16}$
Residuals	138	0.00001560	1.1300×10^{-16}		

Two-way ANOVA on male femur length of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.00	0.0037	0.9321	0.3357
Individual	87	907.88	10.4354	2639.0402	$< 2.2 \times 10^{-16}$
Side X individual	87	0.75	0.0086	2.1792	6.685×10^{-6}
Residuals	176	0.70	0.0040		

Two-way ANOVA on male femur length of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.015	0.0147	2.8158	0.0956
Individual	68	291.053	4.2802	820.0810	$< 2.2 \times 10^{-16}$
Side X individual	68	2.118	0.0311	5.9680	$< 2.2 \times 10^{-16}$
Residuals	138	0.720	0.0052		

Two-way ANOVA on female femur weight of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.0000055	5.4700×10^{-6}	104.486	$< 2.2 \times 10^{-16}$
Individual	65	0.0143430	2.2066×10^{-4}	4215.243	$< 2.2 \times 10^{-16}$
Side X individual	65	0.0000439	6.7500×10^{-7}	12.869	$< 2.2 \times 10^{-16}$
Residuals	132	0.0000069	5.2000×10^{-8}		

Two-way ANOVA on female femur weight of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.0000002	2.0800×10^{-7}	1.0367	0.31
Individual	86	0.0208322	2.4223×10^{-4}	1209.6097	$< 2.2 \times 10^{-16}$
Side X individual	86	0.0000991	1.1530×10^{-6}	5.7559	$< 2.2 \times 10^{-16}$
Residuals	174	0.0000348	2.0000×10^{-7}		

Two-way ANOVA on female femur length of the reference site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.03	0.0302	7.6671	0.006474
Individual	62	1521.32	24.5375	6232.6174	$< 2.2 \times 10^{-16}$
Side X individual	62	0.54	0.0088	2.2280	7.532×10^{-5}
Residuals	162	0.50	0.0039		

Two-way ANOVA on female femur length of the contaminated site frog

Source of variance	df	Sum square	Mean square	F value	P
Side	1	0.06	0.0642	9.745	0.002117
Individual	83	1527.96	18.4092	2796.2066	$< 2.2 \times 10^{-16}$
Side X individual	83	1.13	0.0137	2.0739	3.454×10^{-5}
Residuals	168	1.11	0.0066		

BIOGRAPHY

Acting Sub Lt. Panupong Thammachoti was born on July 31st, 1986 in Bangkok, Thailand. He has graduated a Bachelor of Science degree in Biology with second-class honors from Department of Biology, Faculty of Science, Chulalongkorn University (CU) since 2009. He has then continued his study as a graduate student in Master's degree Program in Zoology, Department of Biology, Faculty of Science, CU with full support from the Human Resource Development in Science Project (Science Achievement Scholarship of Thailand, SAST) since 2009. He also completed a two-week international training course on New Trends and Methodology in Animal Ecology and Conservation Biology from Peking, China in 2011. During his study, he had chance to carry out field surveys in paddy fields at Nan Province for more than a year as part of the CU Centenary Academic Development Plan to encourage graduate student to get a first hand experience in field environment as well as perceiving the country's need in real life situations. During the course of this project, he also has chance to be at the giving end by disseminating the knowledge on natural history of Nan Province to primary and secondary school students in rural areas of Nan Province. In addition to this, he has further acquired field experience in several excursions of the Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG) to survey amphibians and reptiles in Thai Islands and other reserved areas. During his graduate practice, he has given both oral and poster presentations in the international conferences including the 5th International Congress of Chemistry and Environment at Port Dickson, Malaysia in 2010 and the 32nd Annual Meeting of the Society of Environmental Toxicology and Chemistry North America at Boston, M.A., U.S.A. in 2011

Research Publication & Presentation:

-**Thammachoti, P.**, Khonsue, W., Kitana, J., Varanusupakul, P. and Kitana, N. 2012. Morphometric garavimetric of the rice frog *Fejervarya limnocharis* living in areas with different agricultural activity. *Journal of Environmental Protection*. 3(10): xx-xx.

-**Thammachoti, P.**, Khonsue, W., Kitana, J., Varanusupakul, P. and Kitana, N. 2011. Herbicide utilization in paddy fields of northern Thailand alter morphometric and gravimetric parameters of populated rice frog *Fejervarya limnocharis*. *Abstract: the 5th International Congress of Chemistry and Environment*, May 27-29, 2010, Port Dickson, Malaysia. p. 108.

-**Thammachoti, P.**, Khonsue, W., Kitana, J., Varanusupakul, P. and Kitana, N. 2011. Influence of herbicides on morphology of rice frog *Fejervarya limnocharis* in paddy fields, Nan Province. *Abstract, the 1st Thai National Symposium on Animal Care & Use for Scientific Purposes*, July 11-13, 2011, Bangkok, Thailand. p. 72.

-**Thammachoti, P.**, Khonsue, W., Kitana, J., Varanusupakul, P. and Kitana, N. 2011. Influence of atrazine utilization in paddy field on morphology of the populated rice frog *Fejervarya limnocharis* in Thailand. *Abstract: The Society of Environmental Toxicology and Chemistry North America 32nd Annual Meeting*, 13-17 November, 2011, Boston, M.A., U.S.A. p. 297.

-**Thammachoti, P.**, Khonsue, W., Kitana, J., Varanusupakul, P. and Kitana, N. 2012. Impact of herbicide utilization on morphology of populated rice frog *Fejervarya limnocharis* in paddy fields, Nan Province. *Abstract, the 1st National Symposium on Biodiversity Management*, February 2-3, 2012, Nonthaburi, Thailand. p. 92.