INFLUENCES OF HERBICIDES ON HEALTH OF RICE FROG
Fejervarya limnocharis POPULATIONS IN NAN PROVINCE, THAILAND

Mr. Luhur Septiadi

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อิทธิพลของสารฆ่าวัชพืชต่อสุขภาวะของประชากรกบหนอง
Fejervarya limnocharis ในจังหวัดน่าน ประเทศไทย

นายลูฮูร์ เซฟไทดิ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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Thesis Title	INFLUENCES OF HERBICIDES ON HEALTH OF RICE FROG *Fejervarya limnocharis* POPULATIONS IN NAN PROVINCE, THAILAND
By	Mr. Luhur Septiadi
Field of Study	Zoology
Thesis Advisor	Assistant Professor NOPPADON KITANA, Ph.D.
Thesis Co Advisor	PANUPONG THAMMACHOTI, Ph.D. Maître de conférences Julien Claude, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

____________________ Dean of the FACULTY OF SCIENCE

(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

____________________ Chairman

(Associate Professor CHATCHAWAN CHAISUEKUL, Ph.D.)

____________________ Thesis Advisor

(Assistant Professor NOPPADON KITANA, Ph.D.)

____________________ Thesis Co-Advisor

(PANUPONG THAMMACHOTI, Ph.D.)

____________________ Thesis Co-Advisor

(Maître de conférences Julien Claude, Ph.D.)

____________________ Examiner

(Assistant Professor AMPORN WIWEKWEAW, Ph.D.)

____________________ Examiner

(Assistant Professor PONGCHAI DUMRONGROJWATTHANA, Ph.D.)

____________________ External Examiner

(Mohd Sham Bin Othman, Ph.D.)

ลูฮูร์ เซฟไทดิ : อิทธิพลของสารฆ่าวัชพืชต่อสุขภาวะของประชากรกบหนอง *Fejervarya limnocharis* ในจังหวัดน่าน ประเทศไทย. (INFLUENCES OF HERBICIDES ON HEALTH OF RICE FROG *Fejervarya limnocharis* POPULATIONS IN NAN PROVINCE, THAILAND) อ.ที่ปรึกษาหลัก : ผศ. ดร.นพดล กิตนะ, อ.ที่ปรึกษาร่วม : อ. ดร.ภาณุพงศ์ ธรรมโชติ,ผศ ดร.จูเลียง โคลดเดอะ

การใช้สารฆ่าวัชพืชปริมาณมากในพื้นที่เกษตรอย่างต่อเนื่องอาจสร้างความเสี่ยงทางอนามัยสิ่งแวดล้อมและสุขภาวะของสิ่งมีชีวิตที่ไม่ใช่เป้าหมายรวมทั้งสัตว์สะเทินน้ำสะเทินบก จากงานวิจัยที่ดำเนินการในปี พ.ศ. 2553-2554 โดยใช้กบหนอง *Fejervarya limnocharis* เป็นสัตว์เฝ้าระวัง แสดงให้เห็นการสะสมสารฆ่าวัชพืช ได้แก่ แอทราซีน, ไกลโฟเสต, พาราควอต ในสิ่งแวดล้อมและเนื้อเยื่อตลอดจนผลกระทบต่อสุขภาวะของกบ ในการศึกษาปัจจุบันมุ่งเน้นศึกษาอิทธิพลของสารฆ่าวัชพืชต่อสุขภาวะของประชากรกบหนอง โดยเก็บตัวอย่างกบหนองจากนาข้าว 2 แห่ง ที่มีการใช้สารฆ่าวัชพืชแตกต่างกันในจังหวัดน่าน ประเทศไทย ระหว่างเดือนกรกฎาคม 2563 ถึง กุมภาพันธ์ 2564 เมื่อตรวจสอบการปนเปื้อนสารฆ่าวัชพืชในตัวอย่างน้ำจากพื้นที่เกษตรพบการปนเปื้อนแอทราซีนเฉพาะในพื้นที่ปนเปื้อนและเมื่อตรวจสอบการปนเปื้อนในเนื้อเยื่อกบหนองพบว่ามีสารฆ่าวัชพืชทั้ง 3 ชนิดสะสมในกบหนองจากทั้งสองพื้นที่ โดยพื้นที่ปนเปื้อนมีระดับพาราควอตสูงกว่า เมื่อตรวจสอบพารามิเตอร์ในระดับร่างกายสัตว์พบว่ากบหนองจากพื้นที่ปนเปื้อนมีน้ำหนักรังไข่สูงกว่า ซึ่งอาจแสดงถึงผลจากการได้รับเอสโทรเจนแปลกปลอม มีน้ำหนักตับแตกต่างกันอย่างมีนัยสำคัญแสดงถึงการได้รับสารแปลกปลอม และมีน้ำหนักตัวแตกต่างกันอย่างมีนัยสำคัญ เมื่อศึกษาพารามิเตอร์ระดับประชากร พบว่ามีความแตกต่างอย่างมีนัยสำคัญระหว่างประชากรในด้านรูปแบบการเติบโต, fluctuating asymmetry ของกระดูกรยางค์ 5 ชิ้น และรูปแบบการแจกแจงความถี่ของขนาดตัวกบโดยพบการกระจายแบบไม่ได้สัดส่วนในประชากรจากพื้นที่ปนเปื้อน ซึ่งอาจแสดงถึงผลของสารฆ่าวัชพืชต่อการเติบโต การเจริญ และ โครงสร้างประชากร การที่พบความแตกต่างระหว่างพื้นที่ทั้งด้านระดับการปนเปื้อนสารฆ่าวัชพืช พารามิเตอร์ระดับร่างสัตว์และพารามิเตอร์ระดับประชากรแสดงให้เห็นว่าสารฆ่าวัชพืชอาจมีผลกระทบเชิงลบต่อประชากรกบหนองทำให้เห็นการเปลี่ยนแปลงแบบค่อยเป็นค่อยไปอย่างต่อเนื่องในระบบนิเวศเกษตร ผลการศึกษานี้อาจใช้เป็นสัญญาณเตือนถึงอันตรายเชิงอนามัยสิ่งแวดล้อมต่อสัตว์มีกระดูกสันหลังที่อยู่อาศัยใกล้กับพื้นที่ที่ใช้สารฆ่าวัชพืชรวมทั้งมนุษย์

สาขาวิชา	สัตววิทยา	ลายมือชื่อนิสิต ....
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Luhur Septiadi : INFLUENCES OF HERBICIDES ON HEALTH OF RICE FROG *Fejervarya limnocharis* POPULATIONS IN NAN PROVINCE, THAILAND. Advisor: Asst. Prof. NOPPADON KITANA, Ph.D. Co-advisor: PANUPONG THAMMACHOTI, Ph.D.,Maître de conférences Julien Claude, Ph.D.

Intensive and continuous herbicide use in agriculture may pose a risk to health of environment and non-target organisms, including amphibian. Prior research conducted between 2010-2011 using the rice frog, *Fejervarya limnocharis*, as a sentinel revealed an accumulation of herbicides (atrazine, glyphosate, and paraquat) in environmental samples and frog tissue, as well as adverse effects on the health status. This study aims to determine the potential influence of herbicides on population health of the rice frog. Between July 2020 and February 2021, frogs were collected from two paddy fields with varying degrees of herbicide use in Nan province, Thailand. The results of the herbicide residue analysis in water samples indicated that detectable amounts of atrazine were found only in the contaminated site. These three herbicides were detected in frog tissues from both sites, with the contaminated site exhibiting a higher level of paraquat residue. The results on organismal parameters indicated that frogs from the contaminated sites had a greater ovarian weight, indicating a possible effect of xenoestrogen exposure, a significant difference in liver weight, possibly due to xenobiotic exposure, and a significant difference in body weight. The results on population parameters indicated significant differences in growth patterns, fluctuating asymmetry on five appendage bones of frogs, and size-frequency distribution with disproportionate distribution of the contaminated site population, indicating a possible herbicide effect on growth, development and population structure. Site-related differences in herbicide residue, organismal and population parameters indicate that herbicide use may have adverse effects on the health of the rice frog *F. limnocharis* population, resulting in subtle and persistent changes to paddy field ecosystems. The findings of this study may serve as a warning about potential environmental health hazards for vertebrates that live near herbicide utilization areas, including human.

Field of Study:	Zoology	Student's Signature ....
Academic Year:	2021	Advisor's Signature ....
		Co-advisor's Signature ....
		Co-advisor's Signature ....

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CHAPTER I

INTRODUCTION

Environmental contamination continues to be a significant issue, posing a serious threat to the health of communities worldwide. Contamination occurred as a result of a variety of factors, including industrial pollution and agrochemical pollution. In agricultural sectors, intensive agrochemical use has impacted many people, particularly in rural areas, due to the persistency of the chemicals, which cannot degrade rapidly in nature (Roberts, 1996). The use of agrochemicals may leave residues that are harmful to environment, as well as to organisms and humans living in the vicinity.

Southeast Asia, as one of the world's most populous regions (Worldometers, 2018) and agriculturally productive regions (Liu et al., 2020), is vulnerable to health and environmental hazards as a result of increased use of agrochemicals (Lam et al., 2017). Agrochemicals have the potential to accumulate in the environment, posing a risk to non-target organisms and humans living in the area. These factors may contribute to the adverse effects of environmental contamination observed in a variety of sentinel animals, both invertebrates (Won et al., 2005; Barus et al., 2007) and vertebrates (Golden and Rattner, 2003; Bossart, 2011; Tavalieri et al., 2020).

Sentinel species may provide valuable information about the potential impact of agrochemical contamination. The sentinel species should have a home range in an area that will be monitored, be easily counted, have a stable and large population size, and show measurable responses to the contaminant of concern (National Research Council, 1991). According to these criteria, amphibians serve as excellent sentinel species for agrochemical contamination because xenobiotics can enter their systems via a variety of routes (Rollins-Smith et al., 2006), most notably through their semi-permeable skin (Roy, 2002). Additionally, amphibians are vulnerable and sensitive to environmental change and stressors (Venturino et al., 2003), as they have evolved to live in both terrestrial and aquatic habitats (Duellman and Trueb, 1994). Due to their detectable response to environmental changes (Roy, 2002), numerous studies on amphibians have been conducted using a variety of parameters, including biological response, morphology, and population.

It was previously assumed that environmental contamination contributed to the global decline of amphibians (Blaustein and Wake, 1990; Mann et al., 2009). This claim is supported by studies demonstrating morphological changes caused by herbicide contamination, such as abnormal lengths at metamorphosis and gonad (Osano et al., 2002; Howe et al., 2004), as well as liver damage (Riaño et al., 2020). Additionally, agrochemical contamination may result in increased environmental stressors in the ecosystem, which may have a significant negative impact on the population's growth and survival (Gahl et al., 2011). Previous studies have shown that agrochemical contamination has an effect on growth patterns (Thammachoti et al., 2012; Hegde and Krishnamurthy, 2014), population structure disruption (Lambert et al., 2015), and developmental instability in a variety of amphibian species (Thammachoti, 2012; Zhelev et al., 2015b; Costa and Nomura, 2016; Zhelev et al., 2019). Thus, there are compelling evidences that the intensive agrochemical use may have a detrimental effect on amphibians, as evidenced by organismal to population-level parameters.

Agriculture is a critical sector of the Thai economy, resulting in a high demand for imported pesticides (Laohaudomchok et al., 2020), the majority of which are herbicides. Nan province, located in northern Thailand, is a major agricultural area where imported pesticides were primarily herbicides, such as atrazine, glyphosate, and paraquat (Chanpong, 2008). Atrazine and glyphosate have been detected in environmental samples and frog tissues from contaminated paddy fields in previous studies (Thammachoti, 2012; Jantawongsri et al., 2015). Atrazine is a well-known endocrine disrupting chemical that has been shown to have an effect on the reproductive system of amphibians (Hayes et al., 2006), whereas glyphosate and paraquat have been shown to have an effect on amphibian growth and overall health (Osano et al., 2002; Babalola et al., 2019). It is critical to monitor herbicide contamination and its adverse effect on amphibians that live in these areas. This may also serve as an early warning for the dangers associated with continuous and intensive herbicide use.

Scope of the study

Herbicides (i.e., atrazine, glyphosate, and paraquat) may contaminate paddy fields in Nan Province, Thailand, and may have an adverse effect on non-target organisms that live in these areas. The rice frog, *Fejervarya limnocharis*, was used as a sentinel species in this study because it is abundant in paddy fields and has a stable population, making it susceptible to long-term herbicide contamination exposure. To assess the health of rice frogs, sampling was conducted in paddy fields with different degree of pesticide use during July and October 2020 and February 2021, spanning the seasonal period (wet-dry season) and agricultural cultivation cycle. Multiple analyses were performed on the samples, including herbicide contamination, organismal parameter, and population parameter (Figure 1.1). To determine the extent of herbicide contamination, herbicide residues were analyzed in water and frog tissue samples. To investigate the adverse effects on morphology, gonad weight, liver weight, and body weight were compared between sites. To examine the effect of environmental stressors on populations, the growth pattern, size-frequency distribution, and fluctuating asymmetry of the population were compared between sites.

Hypothesis

There are significant differences in 1) herbicide residues, 2) organismal parameters, and 3) population parameters between rice frogs *F. limnocharis* living in contaminated agricultural area with those from reference agricultural area.

Objectives

1. To examine herbicide residues in water and tissues of rice frogs *F. limnocharis* populations living in agricultural areas of Nan Province, Thailand
2. To examine health of rice frogs *F. limnocharis* populations living in agricultural areas of Nan Province, Thailand based on organismal parameters
3. To examine health of rice frogs *F. limnocharis* populations living in agricultural areas of Nan Province, Thailand based on population parameters

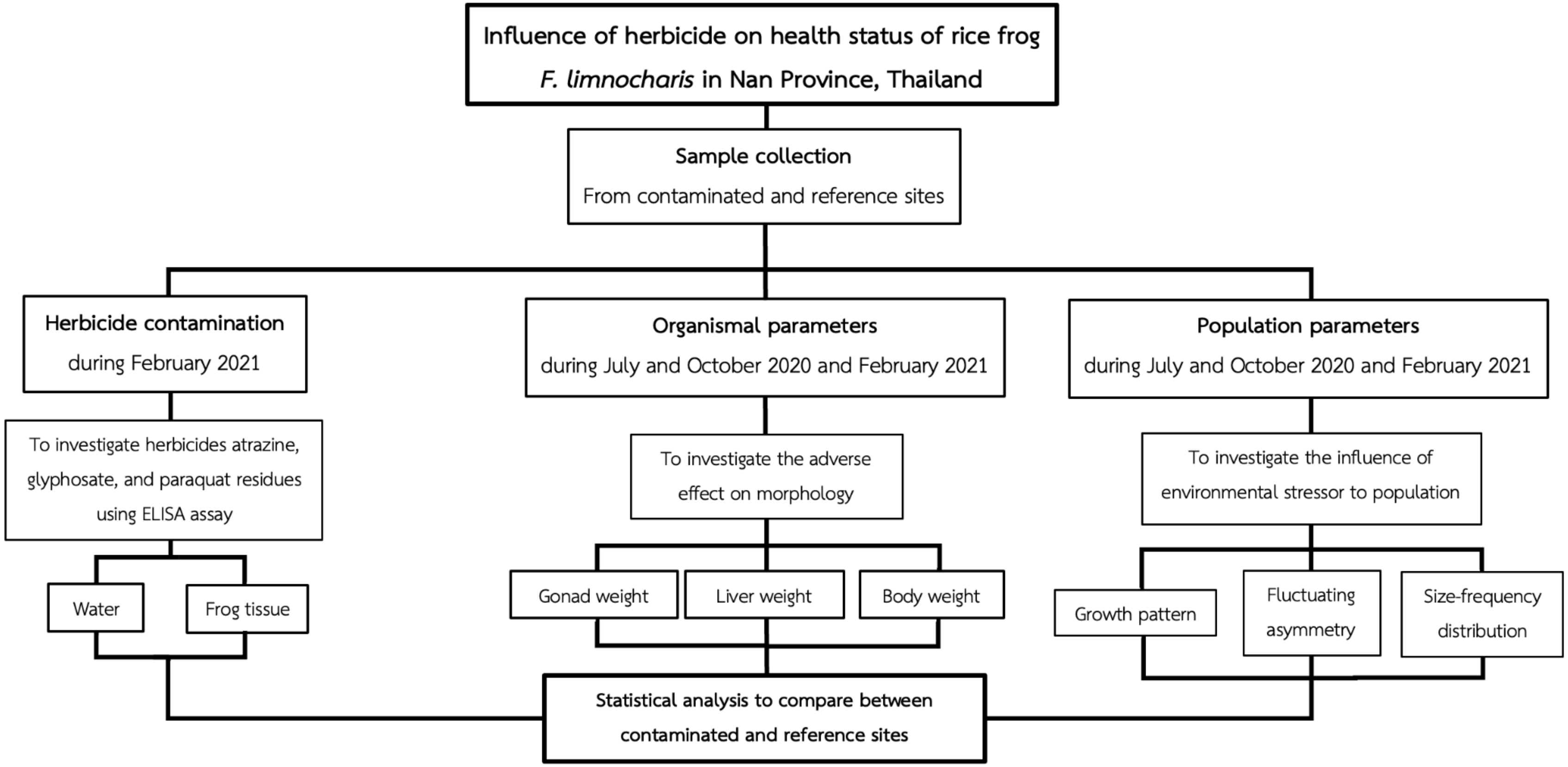

Figure 1.1 Scope of the study: Influences of herbicides on health of rice frog, *Fejervarya limnocharis* populations in Nan Province, Thailand

CHAPTER II
LITERATURE REVIEW

A. Agricultural activity and agrochemical in Nan Province, Thailand

The agricultural sector in Thailand is considered as the foundation of its economy which 30% of the country's population is employed, and up to 70% of the population from rural areas relied on it (Laohaudomchok et al., 2020). Based on the land areas and agricultural activities, a large number of pesticides (e.g., insecticides, fungicides, and herbicides) were used. The most common herbicides that were used are paraquat, glyphosate, 2,4-D, ametrine, and atrazine (Laohaudomchok et al., 2020). Along with the other 2 pesticides (i.e., fungicides and insecticides), the trends of imported pesticides have increased significantly over the past decades. In the northern part of Thailand, one major area that utilizing a different degree of pesticide is Nan province (Figure 2.1). With a total area of 11.472.026 km^2, the province approximately uses 35.23% (404,211.2 hectare) of its total area for the agricultural purposes (Land Development Department, 2018b). Various agricultural activities in these areas include paddy fields, field crops, woody plants, fruit trees, horticulture, swidden, pasture, and livestock (Land Development Department, 2018a).

Majority of the people in Nan province are working as a farmer, particularly in a paddy field. Therefore, they are vulnerable to be exposed to chemical contaminants (Chanpong, 2008). A previous 2008 report showed that the amount of total imported chemicals (i.e., herbicides, insecticides, and chemicals for plant diseases) was 1,274,100 kg with a total valuation of 232.1 million Baht (6.9 million USD) (Chanpong, 2008). Prior studies from 2011–2012 confirmed that the amount of atrazine and paraquat were detected in the water and tissues of animals collected in the paddy field of Nan province (Maneein, 2012; Thammachoti, 2012; Thitiphuree, 2012). Additionally, a recent report in 2016 revealed that out of 21 insecticides, 15 herbicides, and 12 chemicals listed and used for plant diseases by Nan farmers, 12 of those found to contaminated both the soil, water, and plant (Patarasiriwong, 2016). In December 2019, it was decided that the use of paraquat is prohibited (along with tightening restrictions on several pesticides) due to its potential effect on health hazards (Government of Thailand, 2020; Laohaudomchok et al., 2020).

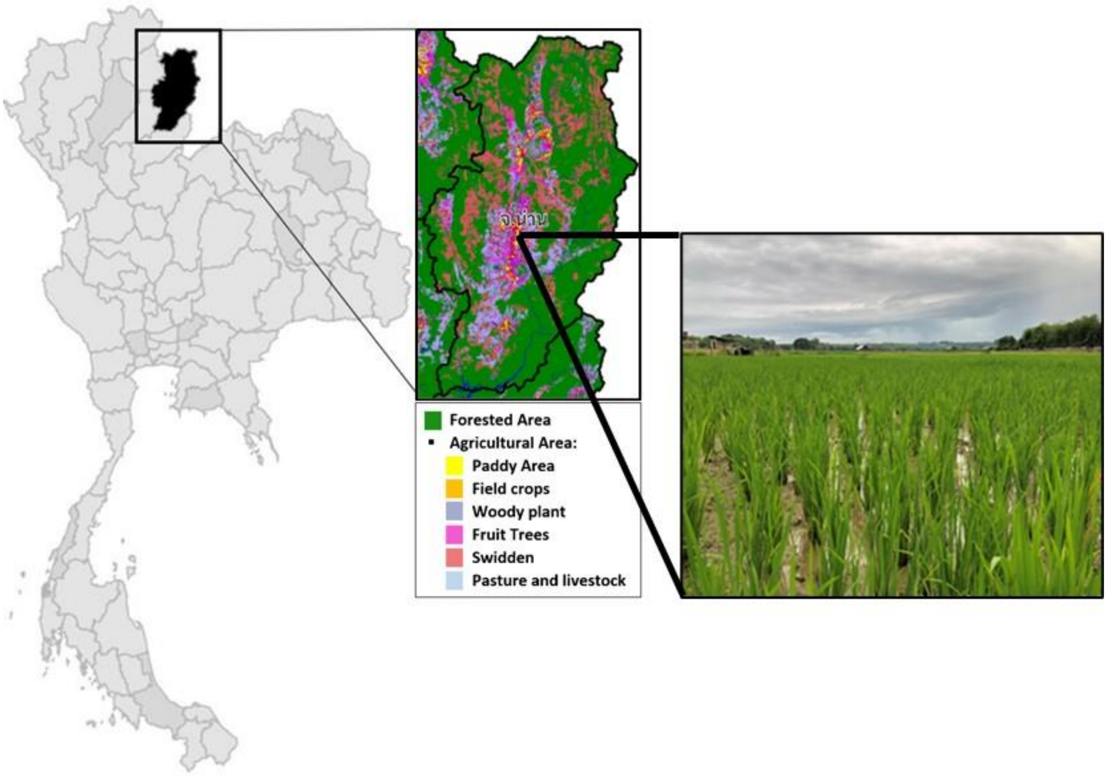

Figure 2.1 Diagram showing agricultural areas in Nan Province, Thailand, modified from Land Development Department (2018b)

Agricultural communities in Nan Province face short growing seasons, high labor costs, and difficult topography (Mambro, 2015). These factors limit the variety of crops that can be grown in Nan Province, resulting in farmers preferring monoculture—repeated planting of a single crop in the same field during the growing season, over polyculture—repeated planting of two or more crops in the same field during the growing season, where corn and rice were the predominant crops planted (Sakchai Korkerd, interview, 1 November 2021; Appendix A).

The agricultural cultivation cycle in Nan Province is highly dependent on the availability of water, which is obtained and channeled from the province's adjacent major river or from a smaller stream that originates from the adjacent mountain (Panupong Thammachoti, interview, 1 November 2021). Here, weather and seasonal factors play a significant role in agricultural yield success. In Nan Province, Thailand, the weather and seasonal period can be generally divided as early wet season (April to June), late wet season (July to September), early dry season (October to December), and late dry season (January to March) (Appendix B). The intensive utilization of herbicides was usually started before planting season in December–January (late dry season) and and June–July (late wet season) (Appendix A–B).

B. Common herbicides property, environmental fate, and its effect on amphibians

1. Atrazine

Atrazine (IUPAC name: 6-chloro-4-N-ethyl-2-N-propan-2-yl-1,3,5-triazine-2,4-diamine) is systemic triazine herbicide (Figure 2.2; Table 2.2) (U.S. Environmental Protection Agency, 2003a). It is a common herbicide used to control the pre-emergence of broadleaf weed by inhibiting photosynthesis of target plants through competition with plastoquinone II at its binding sites in the electron transport process in photosystem II (Devine et al., 1992; Singh and Jauhari, 2017). It is also known as the endocrine-disrupting chemicals (EDCs), capable of interrupting the hormonal sex regulations in frogs (Hayes et al., 2006). As the most generally used herbicide in the USA, atrazine has received a critical review for its toxicity, maximum contamination level, degradation, and potential effect on many vertebrates including amphibians (U.S. Environmental Protection Agency, 2003b).

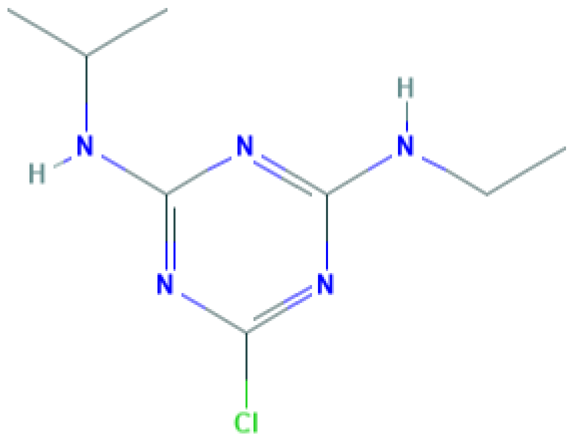

Figure 2.2 Chemical structure of atrazine (National Center for Biotechnology Information, 2021a)

Atrazine does not occur naturally in the environment, it is odorless, colorless, and commonly present in surface water and groundwater (Laohaudomchok et al., 2020). In soil, atrazine is strongly assimilated to sediment and could be degraded by microbial decomposition in oxygenated conditions, thereby, susceptible to degradation in soil, with a half-life of 330 days in anaerobic condition and 3–4 months in aerobic condition (U.S. Environmental Protection Agency, 2003a). It is not rapidly degraded on the plant due to its resistance to abiotic hydrolysis. However, the low adsorption of atrazine in the soil indicates that it can leach to ground or surface water. In water, atrazine is more persistent to direct aqueous photolysis and abiotic hydrolysis both in surface and groundwater due to the s-triazine ring that resulting in resistance to microbial degradation (Howard, 1991; Solomon et al., 2008). In contrast, atrazine has a relatively long half-life up to 578 days in anaerobic conditions (U.S. Environmental Protection Agency, 2003a). Atrazine can be degraded in the environment to various degradation products: hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-1,3,5-triazine, HA); deethylatrazine (2-chloro-4-amino-6-ethylamino-1,3,5-triazine, DEA); deisopropylatrazine (2-chloro-4-ethylamino-6-amino-1,3,5-triazine, DIA) and desethyldesisopropylatrazine (2-chloro4,6-diamino-1,3,5-triazine, DEDIA) (Barchanska et al., 2017), which are also considerably persistent and toxic (Hu and Cheng, 2013). Inhalation was the most common route of exposure to atrazine, both in the general population and in occupational exposures. In agricultural areas where atrazine has been applied to crops, the most common route of exposure for the general population is through ingestion of contaminated well water. For dermal exposure, it was deemed to be a minor route of exposure (Agency for Toxic Substances and Diseases Registry, 2003).

There is conflicting evidence of atrazine potential toxicity to amphibians. Studies from Hayes et al. (2002) reveal that atrazine can alter the development and reproduction of *Xenopus laevis* even at the relatively low ecologically relevant dose. Further studies showed that the exposure of herbicides induces complete feminization and chemical castration in male *X. laevis* (Hayes et al., 2010). Recently, a study found the shifted sex ratios of *Acris blanchardi* suggesting that amphibians are sensitive to the exposure of atrazine (Hoskins and Boone, 2018). Previous studies concluded that atrazine causes significant mortality in tadpoles and affecting reproductive health of adult *X. laevis* (Rimayi et al., 2018). It was proposed that atrazine is responsible for inducing the aromatase activity, an enzyme that is responsible to convert testosterone to estrogen in the vertebrates, and may as well be regarded as endocrine-disrupting chemicals (EDCs) (Hayes et al., 2006; Fan et al., 2007). In contrast, a study from Carr et al. (2003) showed that relevant concentrations of atrazine do not affect the metamorphosis, sex ratio, and larynx growth in *X. laevis*. A study observing the gonadal gross morphology, histology, and morphology in *Rana pipiens* also showed that atrazine was not significantly correlated with the hermaphroditism, and they suggest that the caused effect is more to natural processes in development than atrazine exposure (Murphy et al., 2006). Quantitative weight of evidence analysis on the adverse effect of atrazine across several vertebrates' classes shows that atrazine does not adversely affect fish, amphibians, and reptiles (Hanson et al., 2019).

2. Glyphosate

Glyphosate (IUPAC name: N-(phosponomethyl) glycine) is an aminophosphonic analogue (Figure 2.3; Table 2.2) (U.S. Environmental Protection

Agency, 1993a) also known for its trademark names such as Round up®, Rodeo®, and Shackle®. It is commonly used to suppress annual and perennial weeds due to its broad-spectrum and non-selective properties (Govindarajulu, 2008). Glyphosate prevents the essential aromatic amino acids synthesis by inhibiting the 5-enolpyruvil shikimate-3-P synthetase enzyme in plants or microorganisms (Devine et al., 1992). Even though glyphosate has relatively low toxicity to non-target organisms, several studies have reported the adverse effect caused by glyphosate in aquatic animals including amphibians (de Brito Rodrigues et al., 2019; Thanomsit et al., 2020)

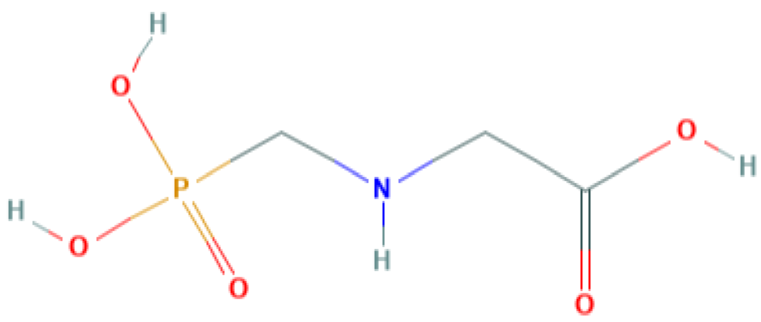

Figure 2.3 Chemical structure of glyphosate (National Center for Biotechnology Information, 2021b)

Glyphosate is tightly and immediately bound to the soil but highly soluble in water (Laohaudomchok et al., 2020). It even reported that it would not move below 15 cm within the soil layer (U.S. Environmental Protection Agency, 1993a). Since glyphosate is an organophosphorus herbicide (carbon-phosphorus bond), consequently, it is resistant to hydrolysis, thermodecomposition, and photolysis degradation (Moore et al., 1983). However, glyphosate can be inactivated by adsorption in clay and degraded in soil by microorganisms to aminomethylphonic acid derivative which they utilized it as carbon, nitrogen, and phosphorus sources (Petit et al., 1995). Glyphosate is generally degraded in soil with a half-life of 7–14 days, and in water with a longer half-life of 60 days (Petit et al., 1995). Nonetheless, glyphosate can contaminate the surface water and resistant to hydrolysis and aqueous photolysis (U.S. Environmental Protection Agency, 1993a). On or near agricultural land, exposure to glyphosate released into the atmosphere may occur via inhalation. It is the primary route of exposure for those who live in close proximity to agricultural land. The general public may be exposed to low levels of glyphosate through consumption of foods containing glyphosate residues and/or contaminated water. For workers, dermal contact is a possible route of exposure. Glyphosate may also persist in soil, increasing the risk of dermal exposure (Agency for Toxic Substances and Diseases Registry, 2020).

The adverse effect of glyphosate on amphibians has been reported based on morphology, genetic, and behavior parameters. In morphology, a study on *R. pipiens* tadpoles reveals a decrease in length at metamorphosis and abnormalities on tail and gonad under the glyphosate exposure (Howe et al., 2004). Glyphosate has been reported to significantly increases melanomacrophagic center in the liver of *Leptodactylus latrans* tadpole (Bach et al., 2018) and induces liver alteration in *Dendropsophus molitor* (Riaño et al., 2020), suggesting liver damages. Another study found that Roundup® can cause extremely high rates of mortality to amphibians (Relyea, 2005). Another study showed that glyphosate formulations could potentially interrupt physiological health, particularly in teratogenicity and growth disruption in *X. laevis* (Babalola et al., 2019). In addition, acute lethal and sublethal effects of glyphosate were performed on *L. latrans* and revealed a detrimental effect on growth, development, and induced morphological abnormalities (Bach et al., 2016). In cellular and genetic tests, it was reported that glyphosate formulation causes cytotoxic and genotoxic effects on neonates of *Eleutherodactylus johnstonei* (Meza-Joya et al., 2013). Other studies found chromosomal aberrations observed on *Sylvirana nigrovittata* after the intramuscular injection with glyphosate (Ruksachat et al., 2021). The glyphosate-based herbicides also potentially alter the mRNA profile on *Lithobates sylvaticus* (Lanctot et al., 2014). In a behavioral context, a mesocosm experiment on *R. dalmatina* tadpoles showed increases hiding activity mimicking similar behavior to those induced by predators (Mikó et al., 2017).

3. Paraquat

Paraquat (IUPAC name: 1,1’-diethyl-4,4’-bipyridinium dichloride) is a bipyridylium herbicide (Figure 2.4; Table 2.2) (U.S. Environmental Protection Agency, 1993b) and one of the most extensively used herbicides in the world (Ronald, 1990). It is also known by several trademark names such as Gramoxone®, Destrone®, and Herboxone®. It is effectively destroying green plant tissues or cell membranes by inhibiting the photosynthesis and respiration process (Haley, 1979) for controlling aquatic weeds. This compound is considered highly toxic, with many reports on organ toxicities and developmental abnormalities in vertebrates, including human (Eftekhari et al., 2020; Kim and Kim, 2020)

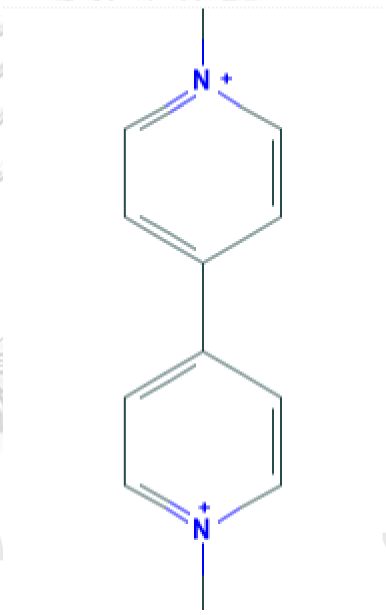

Figure 2.4 Chemical structure of paraquat (National Center for Biotechnology Information, 2021c)

Paraquat is strongly bound to soil particles and sediments due to their cationic properties but less likely to transport to surface water (Laohaudomchok et al., 2020), and hardly decomposed within several years except in surface soils (Ronald, 1990). Paraquat is strongly adsorbed in the soil and tends to remain stable in an inactive state (Eisler, 1991), having a half-life of more than 20 years in soil

(Watts, 2011). It is primarily decomposed by soil microorganisms and mostly photo decomposed through UV light. In water, paraquat is also photodegraded by UV light and aquatic microorganisms. However, it has been reported that the half-life of paraquat in water was estimated to range between 2–820 years depending on the depth of the water and sunlight intensity (Watts, 2011). Paraquat can enter the body when swallowing, breathing, or by contact with the skin or eyes. The main route of exposure in agriculture is through the skin. It occurs through splashing during preparation of spray and its transport, or walking through sprayed vegetation (Watts, 2011).

The adverse effect of paraquat in amphibians has been observed based on toxicity, teratogenic, and reproductive effects. Embryotoxic effects of paraquat has been tested in *X. laevis* (Dial and Bauer, 1984; Vismara et al., 2000; Osano et al., 2002) which reveals growth retardation, multiple tail malformations, reduced head development, and reduced motoric ability. A study on *Scinax nasica* tadpoles shows that corresponding lethal concentration can induce internal gill alteration (Lajmanovich et al., 1998). While the embryotoxic effect is ascertained, the teratogenic effect continues to be debated due to a lack of explanation for the underlying cause. However, the studies by Osano et al. (2002) reveal a drastic increase in mortality, malformation, and growth reduction in *X. laevis* tadpoles confirming the teratogenic effects of paraquat on amphibians. In contexts of reproductive effects, an *in-vitro* study discovered that paraquat affects steroidogenesis in the gonads of *Rana esculenta* (Quassinti et al., 2009).

Table 2.1 Summary of property and toxicity of common herbicides in Thailand

Property and toxicity	**Atrazine**	**Glyphosate**	**Paraquat**
Empirical formula	$C_8H_{14}ClN_5$[A]	$C_3H_8NO_5P$[C]	$C_{12}H_{14}Cl_2N_2$[B]
CAS registry number	1912-24-9[A]	1071-83-6[C]	4685-14-7[B]
Melting point	173 –175°C[A]	230°C (decomposes)[C]	300°C (decomposes)[B]
Solubility in water	33 mg/L at 25°C[A]	10.5 g/L at pH 1.9 and 20°C[C]	6.2 x 10^5 mg/L at 20°C[B]
Compound	Triazines[A]	Aminophosphonic analogue[C]	Bipyridinium[B]
Acute toxicity	• Amphibian: LC_{50} in *Bufo americanus* > 48 mg/L (8 days)[D] • Fish: LC_{50} in *Pimephales promelas* > 6.000 µg/L (7-day)[D] • Rat: LD_{50} in rats (oral) 1.869–2.080 mg/kg[D]	• Amphibian: LC_{50} in *Crinia insignifera* metamorph > 42.1 mg/L (48 hours)[G] • Fish: LC_{50} in *O. mykiss* 8.3 mg/L at 12 °C (24 hours)[H] • Rat: LD_{50} in rats (oral) 4.320 mg/kg[I]	• Amphibian: LC_{50} in *Limondynastes peronii* 100 mg/L (96 hours)[F] • Fish: LC_{50} in *Oncorhynchus mykiss* 15–32 mg/L[F] • Rat: LD_{50} in rats 95–174 mg/kg[F]
Half-lives in water	578 days (anaerobic aquatic study)[E]	60 days (biodegradation in soil)[L]	2–820 years (depending on sunlight and water depth)[J]
Half-lives in soil	330 days (sediment, anaerobic aquatic study)[E] 3–4 months (aerobic laboratory)[E]	7–14 days[K]	> 20 years[J]
Maximum contaminant level in drinking water	0.003 mg/L[M]	0.7 mg/L[M]	–
Maximum residue limit in foods	0.04 mg/kg (Poultry meat)[N] 0.04 mg/kg (Milks)[N] 0.04 mg/kg (Eggs)[N]	0.05 mg/kg (Poultry meat)[O] 0.05 mg/kg (Milks)[O] 0.05 mg/kg (Eggs)[O]	0.005 mg/kg (Poultry meat)[O] 0.005 mg/kg (Milks)[O] 0.005 mg/kg (Eggs)[O]

Remarks:

References abbreviations as follows: (A) National Center for Biotechnology Information (2021a), (B) National Center for Biotechnology Information (2021c), (C) National Center for Biotechnology Information (2021b), (D) Solomon et al. (2008), (E) U.S. Environmental Protection Agency (2003a), (F) Ronald (1990), (G) Govindarajulu (2008), (H) Folmar et al. (1979), (I) Birch (1993), (J) Watts (2011), (K) Giesy et al. (2000), (L) Petit et al. (1995), (M) U.S. Environmental Protection Agency (2009), (N) Health Canada’s Pesticides & Pest Management (2011), (O) Codex Alimentarius (2006)

4. Amphibian as a sentinel species

Animal sentinels are defined (Stahl, 1997) as any non-human organism that can react to an environmental contaminant before it impacts humans. This definition has provided a much wider research venue to the concept and our response to environmental hazards. The sentinel species is often associated with the term such as indicator species and surrogate species. However, the terms were further delimited, and Stahl (1997) defined the indicator species as an organism that responds to contamination in the environment through scientifically justified methods, whereas surrogate species is defined as a tested organism to replace other organisms for particular reasons.

Prior to the introduced concept of sentinel species, miners have already used canaries as a sentinel of potentially lethal poisonous gases (National Research Council, 1991). From this earliest example, the use of mammalian and (later) non-mammalian sentinel species provides useful information to predicts the potential impact on human health. In general, sentinel species are required to have a measurable response to factor, observable home range in an area to be monitored, easily enumerated, have a stable population number, and occurs abundantly (National Research Council, 1991). As our understanding of the applicability of sentinel species from various animals has been expedited (Stahl, 1997; van der Schalie et al., 1999; Roy, 2002; Bossart, 2011) along with the imminent threat of agrochemicals hazards to the environment (Carvalho, 2017), it is recommended to use a sensitive and susceptible group of animals to specific environmental stress, i.e., amphibians (Roy, 2002).

Amphibians are reasonably ideal sentinel species based on the following evidence: enormous diversity, a wide range of habitats, tolerance to various temperature and oxygen, shared similarities to other vertebrates, and easier to manage (Burggren and Warburton, 2007). The most compelling features that make amphibians regarded as an excellent sentinel organism is their susceptibility and sensitivity to environmental change and stressor, especially chemical contaminants during their complex life cycles living in both aquatic and terrestrial habitats (Duellman and Trueb, 1994; Venturino et al., 2003). Agrochemicals can be absorbed into the body dermally due to their skin's high permeability. This is in addition to the more common routes of pollutant exposure, such as oral ingestion and inhalation. As a result, there are numerous significant routes for agrochemicals to enter the body. Amphibians have been used for study on environmental exposure (Roy, 2002), an animal model for endocrine disrupter study (Kloas and Lutz, 2006), a model organism for environmental genotoxicity (Burlibaşa and Gavrilă, 2011), and other physiological studies (Burggren and Warburton, 2007). In conclusion, the adverse effect on amphibians may occur in other vertebrates, including humans, because of the structure and shared functional similarity. Amphibians, as a non-target organism, may provide a forewarning of the dangerous impact of agrochemical contaminants (Kaiser, 2001).

5. Effect of pesticides on morphology and population of amphibians

Pesticides have been linked to the amphibian population decline (Sparling et al., 2001; Davidson, 2004; Brühl et al., 2011) and considered as one of the major incriminated factors that cause detrimental effect to the survival and growth of amphibians in the world (Baker et al., 2013). Under the bioaccumulation (gradual accumulation of substance), and biomagnification (chemical build-up within the hierarchical food chain) processes (di Rosa et al., 2005; Wu et al., 2009), the impact of pesticides is reflected in various biomarkers, covering both in morphology and population levels.

On a morphological level, atrazine (herbicides) was reported to induce demasculinization-feminization (Hayes et al., 2006; Hayes et al., 2011) and chemical castration in male *X. laevis* (Hayes et al., 2010). While other herbicides (i.e., atrazine, glyphosate, paraquat) were also reported to reduce overall health and increase liver damage in *Fejervarya limnocharis* from Thailand (Thammachoti, 2012; Thammachoti et al., 2012). Similar to the observation of *F. limnocharis* (Hegde and Krishnamurthy, 2014) and several species of common frogs from western Ghats, India (Hegde et al., 2019), the adverse effect could potentially influenced by the presence of agrochemicals. Another study on *Pelophylax ridibundus* also reveals a lower health status probably linked to the intensive use of pesticides and fertilizer (Zhelev et al., 2017). Some abnormalities in *Euphlyctis cyanophlyctis*, such as deformity (micromelia) and gas bubble disease, were observed from urban wetland that has been contaminated by agrochemicals (Jilani et al., 2018). The proximity to pollution sources also shows a significant increase of limb malformations in several amphibians species (Taylor et al., 2005).

On a population level, the environmental stressors (i.e., agrochemicals) have been linked to the disruption of population structure and developmental instability in populations. For instance, common herbicides (i.e., atrazine, paraquat, glyphosate) could increase of fluctuating asymmetry—a proxy of developmental instability in the population, observed in the *F. limnocharis* populations from Nan Province, Thailand (Thammachoti, 2012) and the Western Ghats, India (Hegde and Krishnamurthy, 2014). A similar result in *P. ridibundus* population also reveals an increase of fluctuating asymmetry, potentially due to the intensive use of pesticides and fertilizers (Zhelev et al., 2017). The presence of endocrine-disrupting chemicals (EDCs) (e.g., atrazine) could induce demasculinization-feminization in frogs (Hayes et al., 2011), thereby, altering the structure and sex ratio in the populations. The structure of populations could become female-dominated as previously observed on *R. clamitans* along suburbanization gradient which is frequently contaminated by EDCs (Lambert et al., 2015).

6. Fluctuating asymmetry as proxy of environmental stress on amphibians

Constant internal homeostasis during ontogeny is necessary for an organism to be perfectly symmetrical, which is an ideal form for organism survival (Palmer and Strobeck, 1986). However during development, various internal (genetic) and external (environmental) conditions can negatively affect developmental homeostasis, which may lead to an altered form of phenotype (Palmer and Strobeck, 1986). These factors are known as developmental noise or stress (Figure 2.5) (Palmer and Strobeck, 2003). Subsequently, the theoretical phrase perfect bilateral symmetry is inaccurate in practice as perfection hardly exists, and all developmental processes have some degree of randomness. A small but perpetual deviation from this perfect symmetry could lead to developmental instability also known as fluctuating asymmetry (FA) (Palmer and Strobeck, 2003). Fluctuating asymmetry, as a physical measure of developmental instability in bilateral traits (Palmer and Strobeck, 1986), provides an excellent tool to measure the environmental stress in populations. The key assumption, that is the subtle departure from bilateral symmetry must have not come from a genetic predisposition rather genetic or environmental stress (Palmer and Strobeck, 1992).

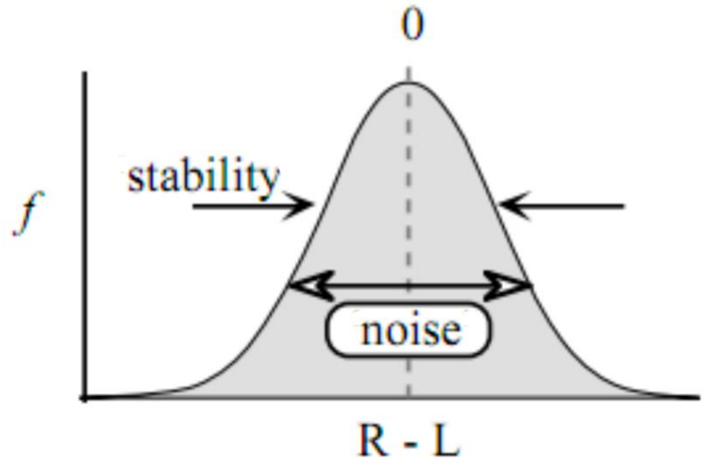

Figure 2.5 Frequency distribution of bilateral symmetry (right and left) influenced by two developmental processes: developmental stability and developmental noise (Palmer, 1994). Abbreviations: (f) frequency, (R – L) right minus left distribution

The reliability of FA has been tested on a population of a variety of amphibians exposed to various degrees of environmental stress. Some researchers reported a weak correlation between environmental stress and FA, e.g., higher FA was not significantly observed on *Physalaemus cuvieri* from urbanized populations (Eisemberg and Bertoluci, 2016), FA was not significantly observed in *Leptodactylus macrosternum* and *Scinax x-signatus* from agricultural environment (Gondim et al., 2020). However, the vast majority of asymmetry studies on amphibians indicate a strong correlation between environmental stress and FA, e.g., logging activity promote higher FA in *Crinia signifera* (Lauck, 2006), *Rana arvalis* shows a higher FA when exposed to an acid environment (Söderman et al., 2007), habitat loss is correlated with the increased of FA in *Bokermannohyla saxicola* (Eterovick et al., 2016), Roundup® pesticides induce higher FA on the tadpole of *P. cuvieri* (Costa and Nomura, 2016), higher FA was observed on *Pelophylax ridibundus* exposed to anthropogenic pollution (Zhelev et al., 2015a) and polluted river (Zhelev et al., 2019); higher FA was observed on *F. limnocharis* from the paddy field with intensive agrochemicals utilization (Thammachoti, 2012). Overall, weight of evidence suggests that fluctuating asymmetry analysis is an effective indicator of environmental stress.

7. Rice Frog *Fejervarya limnocharis*

Rice frog, *Fejervarya limnocharis* (Gravenhorst, 1829), is known by many names e.g., Asian grass frog, common pond frog, field frog, grass frog, and Indian rice frog. It can be found abundantly in paddy fields or wetlands (Iskandar, 1998). The species is currently listed as Least Concern by IUCN due to its very wide distribution, stable populations, and tolerance to a broad range of habitats (van Dijk et al., 2004; AmphibiaWeb, 2021).

Figure 2.6 The rice frog *Fejervarya limnocharis* (Gravenhorst, 1829)

The general characteristic of rice frogs is as follows: small-size (42–46 mm); dorsum covered by longitudinal fold and irregular bumps; ventrum smooth except on groin having granular; snout pointed; small tympanum, half to two-thirds of eye diameter with ridge above; fingers not webbed, first finger longer than second; subarticular tubercles small and prominent; toes fully webbed, tips slightly swollen; dorsum color varies from olive green to reddish-brown, dark V-shaped mark in interorbital, and light green/ yellow stripes crossing through the vertebrae; bumps and ridges often darker than base; ventrum color uniformly white, but yellow and marbled with black in groin. Sex is easily differentiated based on darker vocal sac present in males (absent in female) (Iskandar, 1998; Chan-ard, 2003; AmphibiaWeb, 2021).

The rice frog *F. limnocharis* has a broad range of distribution (Figure 2.7). It is considered to be known only from northern and central groups of Nicobar Islands (India), Indonesia, Malaysia, Laos, Myanmar, Thailand south of the Isthmus of Kra, Cambodia, and Vietnam (Frost, 2021).

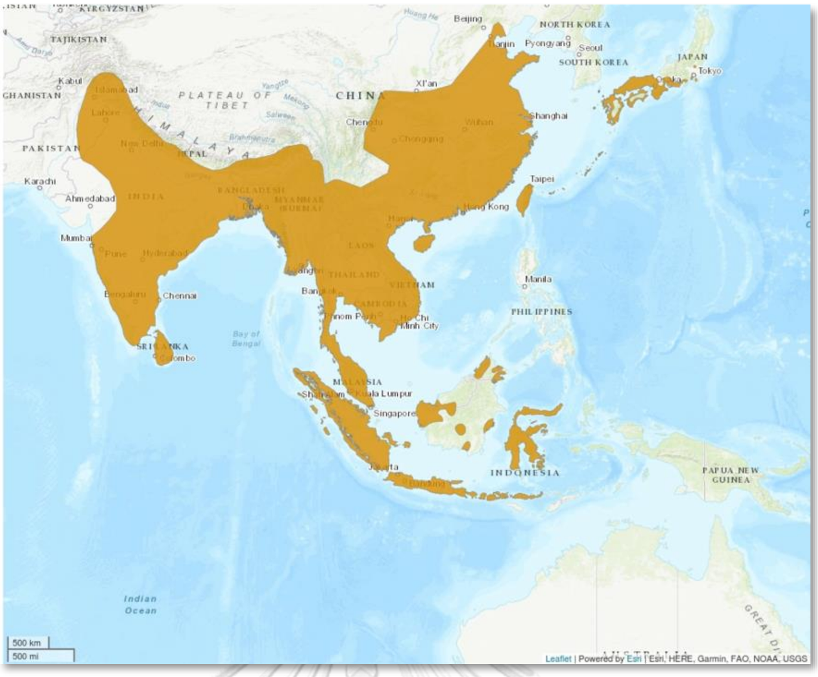

Figure 2.7 The distribution range of rice frog *Fejervarya limnocharis* (van Dijk et al., 2004)

Almost all populations now considered to be members of *Fejervarya* were priorly treated as *F. limnocharis*, hence, the systematics of the rice frog is highly provisional. Toda et al. (1998) reported on sympatric cryptic species under this name from Java and noted the possibility of cryptic species in the Southeast Asia region. Sumida et al. (2007) reported that the *F. limnocharis* comprises of several species including the distinct species from Sri Lanka, India, Taiwan, Ryukus (Japan), and Thailand. Islam et al. (2008) and Hasan et al. (2014) suggested that there are (at least) three species previously referred to *F. limnocharis* occurs in Bangladesh and Myanmar. Several species have been recognized as distinct from their former (*F. limnocharis)* including *F. iskandari, F. vittigera, Minervarya andamanensis, M. agricola,* and *M. nilagrica* (Frost, 2021). The high crypticity of rice frog *F. limnocharis* and overlapping distribution to congenerics highlight that many distinct species remain to be described.

The classification of the rice frog is shown below:

Kingdom: Animalia

Phylum: Chordata

Subphylum: Vertebrata

Class: Amphibia

Superorder: Salientia

Order: Anura

Family: Dicroglossidae

Species: *Fejervarya limnocharis* (Gravenhorst, 1829)

In general, life cycles of rice frogs includes several stages, i.e., egg, tadpole, froglet, juvenile, sub-adult, and adult (Duellman and Trueb, 1994), where abundances may vary according to season and water availability. Based on previous skeletochronological studies, it was reported that the natural lifespan of *F. limnocharis* in Southern India can reached up to 4 years in both sexes (Pancharatna and Deshpande, 2003), slightly different from the subtropical *F. limnocharis* in China, which had a lifespan of 3 years old for males and 4 years old for females (Liao et al., 2011), and slightly longer from tropical *F. limnocharis* from Indonesia, which reached maximum age of 3 years with maximum SVL of 52.37 mm (Phadmacanty et al., 2019). These differences are attributed to many factors such as habitat, altitude, and environmental condition.

The rice frog *F. limnocharis* has been used as a food commodity in Southeast Asia including, Thailand, Laos, and Cambodia (Neang, 2010). It also has been used as sentinel species based on adverse effects on morphology, population, histopathology, and immune response (Othman et al., 2009; Othman et al., 2012; Thammachoti et al., 2012; Nataraj and Krishnamurthy, 2013; Hegde and Krishnamurthy, 2014; Jantawongsri et al., 2015). The rice frog also has been used to analyze the adverse impact of arsenic contamination (Intamat et al., 2016), and nitrate (Krishnamurthy et al., 2008). This further confirms that rice frog *F. limnocharis* is an excellent sentinel species for agrochemical contamination.

CHAPTER III

HERBICIDE RESIDUES IN WATER AND TISSUES OF RICE FROG *Fejervarya limnocharis* POPULATIONS LIVING IN AGRICULTURAL AREAS OF NAN PROVINCE, THAILAND

Introduction

Nan province (northern part of Thailand) is an area with major agricultural activities including paddy fields, maize, field crops, woody plants, fruit trees, horticulture, swidden, pasture, and livestock, where agrochemicals have been used. Previous reports showed that 92% of imported agrochemicals to Nan province were herbicides, i.e., atrazine (6-chloro-4-N-ethyl-2-N-propan-2-yl-1,3,5-triazine-2,4-diamine), glyphosate (N-(phosponomethyl) glycine), and paraquat (1,1'-diethyl-4,4'-bipyridinium dichloride) (Chanpong, 2008). The continuous utilization of agrochemicals may lead to accumulation in the environment which also poses a risk to the non-target organism and humans living in the vicinity. Hence, the extent of contamination in the environment and vertebrates are needed to be monitored. Using sentinel species may provide an early warning of potential adverse effects of agrochemicals contamination to humans.

Amphibians are regarded as good sentinel species of agrochemical contamination since there are many routes that xenobiotics can enter their systems (Rollins-Smith et al., 2006) and due to their observable response to environmental changes (Roy, 2002). Moreover, the most compelling features is their susceptibility and sensitivity to environmental stressor, especially agrochemical contaminants during their complex life cycles living in terrestrial and aquatic habitats (Duellman and Trueb, 1994; Venturino et al., 2003).

Previous studies in agricultural areas at Nan Province reported detectable amounts of atrazine and glyphosate in environmental samples (Jantawongsri et al., 2015), and three herbicides (i.e., atrazine, glyphosate, and paraquat) were found in tissues of frogs (Thammachoti, 2012), rice field crab (Maneein, 2012) and freshwater mussel (Thitiphuree, 2012). Since these herbicides have been intensively used in paddy fields areas of Nan Province, the non-target organisms living in the areas were unavoidably exposed to herbicides. These herbicides are known to exert its adverse effect in amphibians, e.g., atrazine is known as an endocrine-disrupting chemical that can disrupt reproductive system (Hayes et al., 2002; Hayes et al., 2006; Hayes et al., 2010); glyphosate can decrease the growth rate (Howe et al., 2004), damaging the liver (Riaño et al., 2020), and induces morphological abnormality (Bach et al., 2016); and paraquat can induce teratogenic effects (Osano et al., 2002).

In the first segment of this study, herbicide residues were analyzed in composited water samples obtained from paddy fields to investigate the extent of environmental contamination. To investigate the extent of contamination in non-target organisms, rice frog *Fejervarya limnocharis* was used as sentinel species since they are susceptible to long term exposure and accumulation of agrochemicals.

Hypothesis

There are significant differences in herbicide residue in tissues between rice frogs *F. limnocharis* living in contaminated agricultural area with those living in reference agricultural area.

Objective

To examine herbicide residues in water and tissues of rice frogs *F. limnocharis* populations living in agricultural areas of Nan Province, Thailand

Materials and methods

1. Study sites

The study sites are in Wiang Sa District, Nan Province, Thailand. In this region, there are many agricultural areas where herbicides have been utilized for a long time. The potentially contaminated site is a paddy field with intensive herbicide utilization (Yupin Chairaja, personal communication, October 6, 2009) (San Sub-district; 18°30’37.9297” to 18°30’25.6926”; 100°46’24.2638” to 100°46’04.9957”), while the reference site is a paddy field with no history of herbicide utilization for more than 10 years (Srinoon Kamsrikaew, personal communication, October 6, 2009) (Lai-Nan Sub-district; 18°34’35.4157” to 18°34’24.5818”; 100°46’27.5499” to 100°46’12.0212”). The contaminated site locates downstream of the reference site. These sites have similar geographic and climatic conditions but are separated as far as 7 km apart by a major river of Nan province (Figure 3.1).

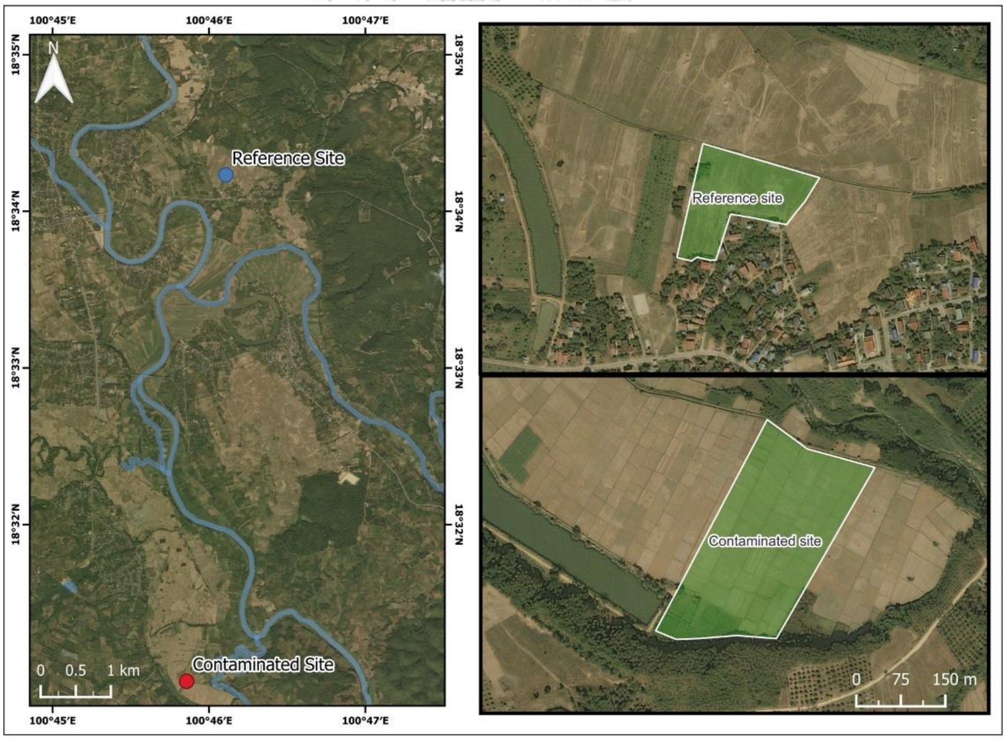

Figure 3.1 Geographical map showing the study sites including contaminated site (San Sub-district) and reference site (Lai-nan Sub-district) in Nan Province, Thailand

For the agricultural practice in these areas, 2-crop cycle was used in the contaminated site, which involved harvesting rice twice a year or growing alternative crops such as corn/sesame during the dry season, and 1-crop cycle was used in the reference site, which involved harvesting rice once a year and leave the farmland undisturbed during the dry season (Sakchai Korkerd, interview, 1 November 2021). This agricultural cycle is highly dependent on water availability. The reference site's paddy field received its water from a small stream adjacent to a nearby mountain, whereas the contaminated site received its water from a nearby major river of Nan Province (Panupong Thammachoti, interview, 1 November 2021; Figure 3.1). It was reported that detectable amount of atrazine (0.15 mg/L) was found in water from the contaminated site, and detectable amount of glyphosate (0.01 mg/kg) was found in sediment from the contaminated site (Thammachoti, 2012). Also, the tissues of rice frogs from the contaminated site had higher residues of herbicides (i.e., atrazine, glyphosate, and paraquat) than those in the reference site (Jantawongsri et al., 2015).

2. Sampling periods

Field sampling was conducted in February 2021 during the late dry season (Appendix B) of the fallow period for the reference site, as well as during the second growing seasons of rice for the contaminated site. As a result, the environment and rice frog tissues should be expected to have a certain level of herbicide contamination.

3. Sample collection

The animal care and use protocol in this study has been approved by the institutional animal care and use committee of the Faculty of Science, Chulalongkorn University (CU-ACUP No. 2123002). Adult rice frog *F. limnocharis* were caught by hand at night during visual encounter survey, and water samples were taken from a series of 4 small patches of water at the paddy field from contaminated site and reference site. The water samples were immediately kept in acetone-rinsed 50 mL conical tube, covered with aluminum foil to avoid sunlight.

Upon arrival in the laboratory at Chulalongkorn University Forest and Research Station, Wiang Sa District, Nan Province, frogs were immediately euthanized by immersion in 0.5% tricaine methane sulfonate solution (Sigma-Aldrich, St. Louis, MO, USA) (Figure 3.2). Frog samples were measured for body weight (BW; g) and snout-vent length (SVL; mm) using Ohaus Pioneer Analytical Balances (accuracy 0.0001 g) and Mitutoyo Absolute Digimatic Caliper (accuracy 0.01 mm), respectively. Samples of frogs were priorly stored in -20°C for herbicides contamination analysis (*n* = 20). Composited water samples were filtered (0.22-micron syringe filters) and placed in acetone-rinsed high density polyethylene plastic containers, wrapped in aluminum foil to avoid exposure to sunlight, and stored at -20°C until further analysis.

Figure 3.2 Rice frogs were euthanized by immersion in 0.5% tricaine methane sulfonate solution.

The frogs were freeze-dried (Epsilon 2-4 LSCplus) until complete dryness (up to 70% weight loss from its initial weight) and measured for the dry weight (g). The dried samples were homogenized with blender until powder-like frog tissue was obtained, then kept in plastic containers with desiccant, covered with aluminum foil to avoid sunlight, and kept at room temperature (25–35°C) until further analysis. Twenty frog tissues samples (contaminated site: *n* = 5 for male, *n* = 5 for female;

reference site: n = 5 for male, n = 5 for female) were extracted for atrazine, glyphosate, and paraquat residues.

4. Herbicide residue analysis in water and frog tissue

To determine the herbicide residue (i.e., atrazine, glyphosate, paraquat), the extraction protocol and enzyme-linked immunosorbent assay (ELISA) procedure were presented for each herbicide. These herbicide residues (ng/g) were also polated to their corresponding initial wet weight (ng/g) for further comparison with the maximum residue limits (Codex Alimentarius, 2006; Health Canada's Pesticides & Pest Management, 2011). The extraction and ELISA protocol were carried out as follows.

4.1. Atrazine

The extraction of atrazine follows the modified protocol of Jacomini et al. (2003). Briefly, 100 mg of lyophilized tissues were mixed with 1 mL of ultrapure H_2O (HPLC Grade; Merck), added with 4 mL of dichloromethane (HPLC Grade; Fisher®), and shaken thoroughly (Vortex-Genie 2). After centrifugation at 1,800 xg for 5 minutes (Wise spin®CF10), 3 mL of organic phase was transferred to a clean glass tube and evaporated under the stream of N_2 gas (TurboVap® II). The residues were reconstituted with 100 µL of methanol and 900 µL of ultrapure H_2O. To check for recovery of extraction, the 2.5 ng of standard atrazine (1 mL of 2.5 ng/mL atrazine solution) was added to a representative sample (as a spiked sample) before proceeding with the subsequent step. Finally, all of the samples were stored at -20°C until further analysis.

ELISA kit for atrazine determination was obtained from Eurofins Abraxis (Warminster, PA), and the methods were followed the manufacturer's protocol. Briefly, 25 µL of assay buffer was added into the wells of a 96-microtiter plate coated with rabbit anti-triazine antibody. Then, duplicated addition of 25 µL of samples, spiked sample, and standard atrazine solution (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 = 5.0 ng/mL) were added into the designated wells. The wells were added with 50 µL of triazine-horseradish peroxidase (HRP)-conjugate solution and incubated on orbital shaker (Mini-Rock shaker PSU-2T BIOSAN) at room temperature (25°C) for 30 minutes. Subsequently, the plate was washed 3 times using washing buffer solution, then blotted for the excess solution. The wells were added with 100 µL of substrate/ color solution (hydrogen peroxide and a chromogen: 3,3’,5,5’-tetramethylbenzidine; TMB), then incubated for another 20 minutes before 50 µL of stop solution (diluted sulfuric acid) was added to each well. The wells were measured for absorbance at 450 nm using microplate reader (Multiskan EX).

To calculate for atrazine concentration, mean absorbance of the duplicated standards/ samples were estimated. Subsequently, %B/B_0 was calculated by dividing the mean absorbance of each standard/ sample with the mean value of atrazine standard S_0 (0 ng/mL). Standard curves were plotted using %B/B_0 for each standard (S_0–S_6) on Y-axis and corresponding atrazine concentration on X-axis. Standard calibration curves were linear (r^2 = 0.9944). Atrazine of each sample could be interpolated using an equation derived from the standard curve: **Y = a ln(X) + b**, where Y is %B/B_0 of the sample and X is the corresponding atrazine concentration in ppb (ng/mL). After taking into consideration on extraction factor, the atrazine concentration in frog tissue was presented in µg/kg dry weight. The limit of detection was 0.96 ng/g dry weight, and the recovery of extraction (spike atrazine 2.5 ng) was 68.00%. The coefficient of variation (CV) for intra-assay precision of atrazine ELISA was 4.98%.

4.2. Glyphosate

The extraction of glyphosate follows the modified protocol of Alferness and Iwata (1994). Briefly, 100 mg of lyophilized tissues were mixed with 200 µL of ultrapure H_2O (HPLC Grade; Merck), added with 100 µL of chloroform (Merck) and 500 µL of 0.1 N HCL, and shaken thoroughly. After centrifugation at 1,000 xg for 10 minutes, 350 µL of aqueous phase was transferred into a clean tube. To check for recovery of extraction, the 0.8 ng of standard glyphosate (200 µL of 4 ng/mL glyphosate solution) was added to a representative sample (as a spiked sample) before proceeding with the subsequent step. Finally, all of the samples were stored at -20°C until further analysis.

ELISA kit for glyphosate determination was obtained from Eurofins Abraxis (Warminster, PA), and the protocol was according to the manufacturer's manual. Briefly, 250 µL of the samples, spiked sample, and standard glyphosate (glyphosate standard 0–5; including: S_0 = 0 ng/mL, S_1 = 0.075 ng/mL, S_2 = 0.2 ng/mL, S_3 = 0.5 ng/mL, S_4 = 1.0 ng/mL, and S_5 = 4.0 ng/mL) were mixed with 1 mL of diluted derivatization reagent, shaken thoroughly, then incubated at room temperature (25°C) for 10 minutes. The duplicated addition of 50 µL derivatized samples, spiked sample, and standard glyphosate were added to designated wells of a 96-microtiter plate coated with goat anti-rabbit IgG antibody. The wells were added with 50 µL of rabbit anti-glyphosate antibody solution and incubated at room temperature (25°C) for 30 minutes. Subsequently, the wells were added with 50 µL of HRP-labeled glyphosate analog solution and incubated at room temperature (25°C) for another 60 minutes. The plate was washed 3 times using washing buffer solution, then blotted for an excess solution. The wells were added with 150 µL of substrate/ color solution (hydrogen peroxide and a chromogen: 3,3',5,5'-tetramethylbenzidine; TMB), then incubated for another 30 minutes before 100 µL of stop solution (diluted sulfuric acid) was added to each well. The wells were measured for absorbance at 450 nm using microplate reader (Multiskan EX) within 15 minutes after the addition of stop solution.

To calculate glyphosate concentration, mean absorbance of the duplicated standards/ samples were estimated. Subsequently, $\%B/B_0$ was calculated by dividing the mean absorbance of each standard/ sample with the mean value of glyphosate standard S_0 (0 ng/mL). Standard curves were plotted using $\%B/B_0$ for each standard (S_0–S_5) on Y-axis and corresponding glyphosate concentration on X-axis. Standard calibration curves were linear (r^2 = 0.9787). Glyphosate of each sample could be interpolated using an equation derived from the standard curve: **$Y = a \ln(X) + b$**, where Y is $\%B/B_0$ of the sample and X is the corresponding glyphosate concentration in ppb (ng/mL). After taking into consideration on extraction factor, the glyphosate concentration in frog tissue was presented in µg/kg dry weight. The limit of detection was 0.564 ng/g dry weight, and the recovery of extraction (spike glyphosate 0.8 ng) was 68.15–90.38%. The coefficient of variation for intra-assay precision of glyphosate ELISA kit were 11.48–20.31%.

4.3. Paraquat

The extraction of paraquat follows the modified protocol of Brown et al. (1996) and Quick et al. (1990). Briefly, 100 mg of lyophilized tissues were priorly mixed with 200 µL of ultrapure H_2O (HPLC Grade; Merck), added with 100 µL of hexane (Merck) and 600 µL of 10% trichloroacetic acid (TCA), and shaken thoroughly. After centrifugation at 2,000 xg for 15 minutes, 500 µL of aqueous phase was transferred into a clean tube as a first extract. The remaining samples were added again with 250 µL of 10% trichloroacetic acid (TCA) and shaken thoroughly. After centrifugation at 2,000 xg for 15 minutes, 200 µL of aqueous phase was transferred into the first extract, thereby, a total extract of 700 µL of aqueous phase was collected. The extracts were added with 400 µL of hexane and shaken thoroughly. After centrifugation at 2,000 xg for 15 minutes, 600 µL of aqueous phase was collected. To adjust the pH to 7.0, extracts were added with 100 µL of 2M Tris-basic buffer and shaken thoroughly. After centrifugation at 2,000 xg for 5 minutes, a total extract of 500 µL of aqueous phase was transferred into a clean tube and labeled as an aqueous extract. The remaining precipitated samples were added with 200 µL of ultrapure H_2O and labeled as a precipitated extract. To check for recovery of extraction, the 1.5 ng of standard paraquat (200 µL of 7.5 ng/mL paraquat solution) was added to a representative sample (as a spiked sample) before proceeding with the subsequent step. Finally, all of the samples were stored at -20°C until further analysis.

ELISA kit for paraquat determination was obtained from Abnova (Thaoyuan City, Taiwan), and the methods were after the manufacturer's protocol. Briefly, duplicated addition of 25 µL of samples, spiked sample, and standard paraquat (paraquat standard 0–5; including: S_0 = 0 ng/mL, S_1 = 0.075 ng/mL, S_2 = 1.25 ng/mL, S_3 = 2.5 ng/mL, S_4 = 3.75 ng/mL, and S_5 = 7.5 ng/mL) were added into the designated wells of a 96-microtiter plate coated with rabbit anti-paraquat antibody. Then, 100 µL of paraquat-HRP conjugate was added to each well and incubated at room temperature (25°C) for 30 minutes. Subsequently, the plate was washed 3 times using washing buffer solution, then blotted for an excess solution. The wells were added with 100 μL of substrate/ color solution (hydrogen peroxide and a chromogen: 3,3’,5,5’-tetramethylbenzidine; TMB), then incubated for another 30 minutes before 100 μL of stop solution (3M HCl) was added to each well. The well was measured for absorbance at 450 nm using microplate reader (Multiskan EX).

To calculate paraquat concentration, mean absorbance of the duplicated standard/samples were firstly estimated. Then, % inhibition was calculated using the formula as follows.

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

Standard curves were plotted using % inhibition of each standard (S_0–S_5) on Y-axis and corresponding paraquat concentration on X-axis. Standard calibration curves were linear (r^2 = 0.9669–0.9966). Paraquat of each sample could be interpolated using an equation derived from the standard curve: **Y = a ln(X) + b**, where Y is % inhibition of the sample and X is the corresponding paraquat concentration in ppb (ng/mL). After taking into consideration on extraction factor, the paraquat concentration in frog tissue was presented in μg/kg dry weight. The limit of detection was 0.10 ng/g dry weight, and the recovery of extraction was 93.31%. The coefficient of variation for intra-assay precision of the paraquat ELISA kit were 18.80–20.21%.

5. Statistical analysis

All parameters were priorly tested for normality and homogeneity of variance. Herbicide residues in rice frogs were analyzed separately between male and female for each site, compared using Student’s t-test or Mann-Whitney rank-sum test, and power of the test analysis. Male and female data were pooled in the absence of a significant sex-related difference. Correlation between corresponding snout-vent length, body weight (initial wet weight), and herbicide residues (i.e., atrazine, glyphosate, paraquat) were determined using Pearson’s correlation and analyzed separately between sexes. Statistical analysis was conducted using Sigma Plot 11.0 and SPSS Stat 28 for MacOS.

Results

1. Herbicide contamination on water

During February 2021, there was a detectable amount of atrazine (1.39 ng/mL) present in the composited water sample from the contaminated site, while levels of atrazine at the reference site, and glyphosate and paraquat at both sites were below limit of detection (0.08 ng/mL for atrazine, 0.083 ng/mL for glyphosate, 0.819 ng/mL for paraquat).

2. Herbicide contamination on frog tissue

Based on February 2021 data, the results showed that there was no significant sex-related difference of herbicide residues between male and female frogs (Appendix C). Therefore, the male and female data were pooled for the analysis.

The results of contamination analysis showed that atrazine, glyphosate, and paraquat was found in tissue of rice frogs in both contaminated site and reference site. There was a higher mean of atrazine residues in frogs from contaminated site than those in reference site, although not significantly. There was a higher mean of glyphosate residues in frogs from contaminated site than those in reference site, although not significantly. The paraquat residues showed a significant site-related difference showing higher mean of residues in frogs from contaminated site than those in reference site (Table 3.1, Figure 3.3).

Table 3.1 Herbicide residues in tissues of rice frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during February 2021

Herbicides (*n* Contaminated: Reference)	Tissue residue in dry weight (ng/g)		Power of test (α= 0.05)
	Contaminated site (Mean ± SEM)	Reference site (Mean ± SEM)	
Atrazine (10:10)[a]	1.60 ± 0.26	1.27 ± 0.24[ns]	0.05
Glyphosate (10:10)[a]	26.05 ± 5.83	6.19 ± 0.61[ns]	0.23
Paraquat (10:10)[a]	115.89 ± 47.11	27.98 ± 8.91[*]	0.29

Remarks:

[a] Compared by Mann-Whitney rank-sum test

[*] Significant difference between sites; $p \leq 0.05$

[ns] No significant difference between sites; $p > 0.05$

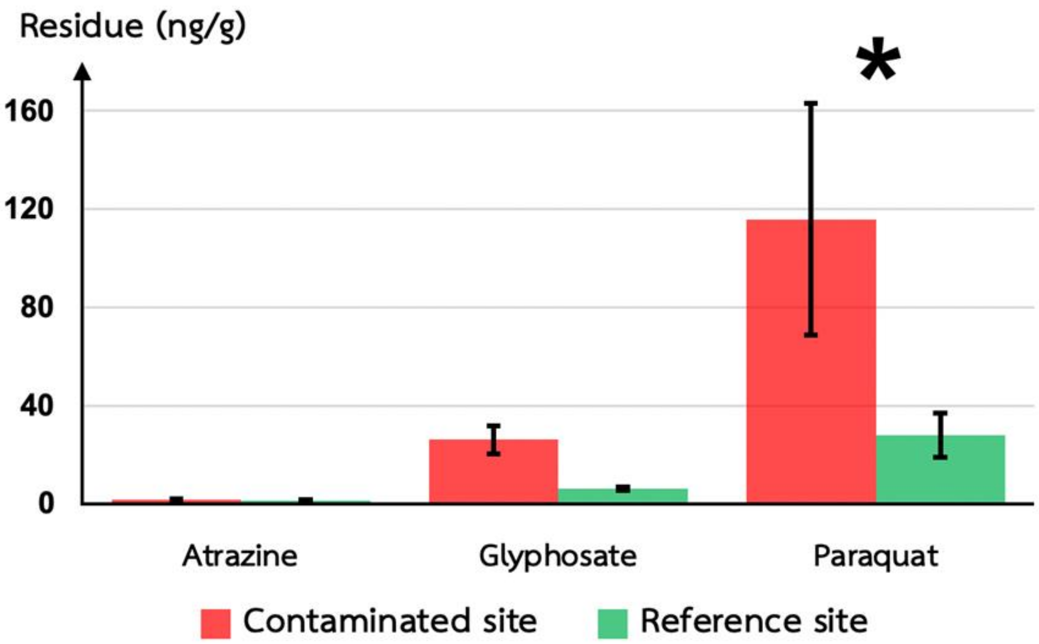

Figure 3.3 Herbicide residues (Mean ± SEM) in tissues of rice frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during February 2021

3. Pearson's correlation analysis between herbicide contamination, body weight, and snout-vent length

Pearson's correlation analysis between snout-vent length, body weight, and herbicides contamination in tissues of adult male frogs showed a positive significant correlation between body weight vs. glyphosate residue. There was no significant correlation between body weight vs. both atrazine and paraquat residues. There was no significant correlation between snout-vent length vs. both atrazine, glyphosate, and paraquat residues (Table 3.2).

Pearson's correlation analysis between snout-vent length, body weight, and herbicides contamination in tissues of adult female frogs showed no significant correlation between snout-vent length and body weight vs. both atrazine, glyphosate, and paraquat residues. There was a significant positive correlation between glyphosate residue vs. paraquat residue (Table 3.2).

Table 3.2 Pearson's correlations coefficients between snout-vent length, body weight, and herbicide residues in tissues of adult rice frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during February 2021

Parameter (n = 10)	Snout-vent length	Body weight	Atrazine	Glyphosate	Paraquat
Male					
Snout-vent length		0.937 $p \leq 0.05$	0.368 $p > 0.05$	0.500 $p > 0.05$	-0.028 $p > 0.05$
Body weight			0.248 $p > 0.05$	0.717 $p \leq 0.05$	-0.141 $p > 0.05$
Atrazine				-0.016 $p > 0.05$	0.442 $p > 0.05$
Glyphosate					-0.247 $p > 0.05$
Paraquat					
Female					
Snout-vent length		0.948 $p \leq 0.05$	-0.087 $p > 0.05$	0.157 $p > 0.05$	0.273 $p > 0.05$
Body weight			-0.045 $p > 0.05$	0.024 $p > 0.05$	0.265 $p > 0.05$
Atrazine				0.509 $p > 0.05$	0.228 $p > 0.05$
Glyphosate					0.737 $p \leq 0.05$
Paraquat					

Discussions

This study found a detectable amount of atrazine in the composited water samples from the contaminated site. However, the level of atrazine was below the Maximum Contaminant Level (MCL) of US EPA in drinking water (0.003 mg/L or 3 ppb) (U.S. Environmental Protection Agency, 2009). It was previously reported that the atrazine level in water from the contaminated site during late dry season in 2011 was beyond the MCL (0.15 mg/L) (Thammachoti, 2012). Still, it cannot be ruled out that there was extensive utilization of atrazine at the paddy field and the relatively low detection of atrazine may be due to the short half-life as it was easily degraded by photolysis or other means of degradations (Wehtje et al., 1981; Scribner et al., 2000), meaning that the herbicides were diluted in water and found in concentration below the detection limit. Even though detected at low concentration, it is noteworthy that atrazine could alter reproductive system of frogs at relatively low concentrations (Hayes et al., 2002). Glyphosate was not detected in the composited water samples possibly due to its degradability upon direct exposure to sunlight and high temperature (Moore et al., 1983; Petit et al., 1995). Paraquat was not detected in water as expected since it was adhered strongly to soil and sediment (Ronald, 1990). As a result, there is still a possibility that these two herbicides contaminated the frogs' tissue via another route of exposure.

There was a limited number of studies reporting herbicides contamination on amphibians, including previous studies on herbicide contamination (i.e., atrazine, glyphosate, and paraquat) on rice frog *F. limnocharis* from Thailand (Thammachoti, 2012). Accumulation of these herbicides could be found on other aquatic vertebrates, including accumulation of glyphosate in carp and tilapia (lethal concentration 50; 5.5–7.9 ppm for 48 hours) (Wang et al., 1994), and accumulation of paraquat in neuromelanin of *Rana temporaria* based on autoradiography (Lindquist et al., 1988). Results from the current study further support that these herbicides could contaminate non-target organisms.

The trend of atrazine and glyphosate concentration is similar to the previous studies during late dry season (January 2011) by having a higher mean of herbicides residues (i.e., atrazine, glyphosate, and paraquat) on frog tissues from contaminated site (Thammachoti, 2012). Interestingly, paraquat residues in the contaminated site were strikingly higher than the reference site (Tables 3.1, Figure 3.3) which is more pronounced than the previous studies (Thammachoti, 2012). The results suggest that three herbicides (i.e., atrazine, glyphosate, paraquat) are, indeed, contaminated the tissue of rice frogs living in the paddy field.

Results on Pearson's correlation analysis (Table 3.2) showed that there was a significant positive correlation between body weight and glyphosate residue in adult male frogs, suggesting that glyphosate could accumulate differently depending on body weight and body condition of the frog. The significant positive correlation between glyphosate and paraquat residues in adult female frogs indicated that those herbicides were concurrently and intensively used during late dry season (February 2021). During this period, it can be argued that the herbicides would be detected on their baseline level due to different agricultural cultivation cycles (Appendix A). Previous studies showed that the highest level of atrazine was found during late wet period of harvest period, whereas glyphosate and paraquat were found at high level during early dry season of post-harvest period (Thammachoti, 2012). Since there was a presence of different degrees of herbicide utilization, the adverse effects on health of rice frogs could be expected.

Rice frog is regarded as delicacies for people, especially from Southeast Asia (Altherr et al., 2011), and the results on herbicides contamination in tissues of frogs raise concern over the consumption of the frogs. It was stated that Good Agricultural Practice (GAP) must conform to the allowed limit of agrochemical residues, thereby, the level of agrochemical residue must be below the maximum residue limit (MRL) allowed in food (Leong et al., 2020). The results showed that atrazine residues (0.193–1.158 ng/g wet weight) were below the ranges of the MRL allowed in food (40 ng/g wet weight) (Health Canada's Pesticides & Pest Management, 2011). Similarly, glyphosate residues (1.286–26.698 ng/g wet weight) were below the MRL allowed in food (50 ng/g wet weight) (Codex Alimentarius, 2006). However, paraquat residues (2.822–148.778 ng/g wet weight) were far beyond MRL allowed in food (5 ng/g wet weight) (Codex Alimentarius, 2006) meaning that the farmers in the areas continue to use banned pesticides (i.e., paraquat) extensively despite the official banned by Thai

Government. This implies that GAP was not followed in the areas, raising concerns about potential health and environmental hazards.

Tightening of pesticide utilization has been a subject of debate in Thailand over the past several years. At present, both chlorpyrifos and paraquat have been officially banned effectively in June 2020 (Government of Thailand, 2020), after the collective movement by Thailand Pesticide Alert Network (Thai-PAN), group of academics from various universities and institutes, and collaborative partners (Rujivanarom, 2018; Wipatayotin, 2018). However, this decision has been opposed by agrochemical industries and several groups of farmers claiming that they will lose profit in agricultural sectors (Tanakasempipat, 2020), and government has not provided alternatives to the banned substances (Taylor, 2020). Nonetheless, any request to lift the ban has been refused by The National Hazardous Substances Committee (NHSC) (Wipatayotin, 2018; Taylor, 2020) and any banned product must be returned. This dispute could influence the seller's decision to reduce the price of the banned pesticides and provoke farmers to apply higher concentrations to empty the stock. Aggravated by the inefficient control of pesticides due to the divided regulatory functions among the governmental regulatory body (Laohaudomchok et al., 2020), consequently, herbicides was utilized indiscriminately. This may explain the high concentration of paraquat in the tissues of rice frogs found during this study (Tables 3.1). This current study identified a high level of paraquat residues, implying intensive utilization of agrochemicals was present. Attempts to reduce pesticide usage through mitigation campaigns such as organic farming, integrated pest management (IPM), good agricultural practice (GAP), and promotion of bio-pesticides must be widely encouraged.

Conclusion

The first part of this study found that atrazine contaminated the waters of paddy fields and that three herbicides (atrazine, glyphosate, and paraquat) contaminated the tissue of rice frogs. Rice frogs from the contaminated site had significantly higher mean herbicide residues than those from the reference site, with paraquat from the contaminated site found above the MRL in food, indicating that it was one of the agrochemicals of concern. Due to the presence of varying degrees of herbicide contamination, detrimental effects on the health of rice frogs could be expected.

CHAPTER IV

HEALTH STATUS OF RICE FROGS *Fejervarya limnocharis* POPULATIONS LIVING IN AGRICULTURAL AREAS OF NAN PROVINCE, THAILAND BASED ON ORGANISMAL PARAMETERS

Introduction

Nan Province is one of the areas in Thailand which have been intensively utilizing agrochemicals for their agricultural activities. The previous study revealed that non-target organisms living in the paddy fields have been exposed to several herbicides, i.e., atrazine, glyphosate, and paraquat (Maneein, 2012; Thammachoti, 2012; Thitiphuree, 2012; Jantawongsri et al., 2015). With the persistence of these agrochemicals, this may pose a risk of having adverse effects on morphology and populations of the non-target organism living nearby the contaminated area, including human.

It is essential to monitor the impact of herbicide contamination by using non-target organisms or sentinel species due to the shared similarities of metabolic process and susceptibility as to humans (Roy, 2002; Venturino et al., 2003). The observed adverse effect on sentinel species may provide forewarning to the danger of continuous and intensive utilization of agrochemicals (National Research Council, 1991).

Amphibians have been considered as the most suitable sentinel species for agrochemical contamination due to their susceptibility and sensitivity to environmental change and stressors (Venturino et al., 2003). Subsequently, studies were reporting the adverse effect of herbicides on the morphology of the amphibians, e.g., abnormality in length at metamorphosis and gonad (Osano et al., 2002; Howe et al., 2004), and liver damage (Riaño et al., 2020), which can pose a threat to their survival. Thus, gonad and liver may be considered as the organs in concern and serves as biomarker of effects. Moreover, a concern was raised on the global decline of amphibians (Davidson, 2004) where one of the underlying factors was agrochemicals contaminations (Gahl et al., 2011), which indirectly affected amphibian survival in the population.

In this segment of the study, the adverse effects of herbicides utilization were investigated on rice frog *Fejervarya limnocharis* since it can be found abundantly in paddy fields, lived in a stable population, making it prone to long-term exposure to herbicide contamination. Using rice frogs as sentinel species, the health status of rice frogs living in paddy fields with different degrees of herbicides utilization were examined based on the gonad weight, liver weight, and body weight, as organismal parameters.

Hypothesis

There are significant differences in gonad weight, liver weight, and body weight between rice frogs *F. limnocharis* living in contaminated agricultural area with those living in reference agricultural area.

Objective

To examine health of rice frogs *F. limnocharis* populations living in agricultural areas of Nan Province, Thailand based on organismal parameters

Materials and Methods

1. Study sites

The study sites are in Wiang Sa District, Nan Province, Thailand. In this region, there are many agricultural areas where herbicides have been utilized for a long time. The potentially contaminated site is a paddy field with intensive herbicide utilization (Yupin Chairaja, personal communication, October 6, 2009) (San Sub-district; 18°30’37.9297” to 18°30’25.6926”; 100°46’24.2638” to 100°46’04.9957”), while the reference site is a paddy field with no history of herbicide utilization for more than 10 years (Srinoon Kamsrikaew, personal communication, October 6, 2009) (Lai-Nan Sub-district; 18°34’35.4157” to 18°34’24.5818”; 100°46’27.5499” to 100°46’12.0212”). The reference site practiced 1-crop cycle, repeated planting of a single crop in the same field throughout the growing season, whereas the contaminated site practiced 2-crop cycle, repeated planting of two or more crops in the same field throughout the growing season, with corn and rice being the most frequently planted crops (Appendix A).

2. Sampling periods

Samplings were conducted during July and October 2020, and February 2021 covering the seasonal period of wet–dry seasons (Appendix B) and agricultural cultivation cycle (Appendix A). Life cycles of rice frogs includes several stages, including egg, tadpole, froglet, juvenile, sub-adult, and adult (Duellman and Trueb, 1994), where abundances may vary according on season and water availability (Appendix B, Appendix D). Field samplings were conducted using purposive sampling method by visual encounter survey (Kusrini, 2019) where several stages of frogs (i.e., froglet, juvenile, sub-adult, adult) were caught by hand at night considering the active time of the species. Extra samples were collected from reference site beyond primary samplings as an additional data for analysis on organismal parameters.

3. Sample collection

The animal care and use protocol in this study has been approved by the institutional animal care and use committee of the Faculty of Science, Chulalongkorn

University (CU-ACUP No. 2123002). Upon arrival in the laboratory at Chulalongkorn University Forest and Research Station, Wiang Sa District, Nan Province, frogs were immediately euthanized by immersion in 0.5% tricaine methane sulfonate solution (Sigma-Aldrich, St. Louis, MO, USA). The samples were counted for the total individuals and corresponding stage for further analysis.

4. Determination on gonad, liver, and body weights

Frog samples were measured for body weight (BW; g) and snout-vent length (SVL; mm) using Ohaus Pioneer Analytical Balances (accuracy 0.0001 g) and Mitutoyo Absolute Digimatic Caliper (accuracy 0.01 mm), respectively. The frogs were dissected and measured for gonad and liver weights with the aid of stereomicroscope (Carl Zeiss) and Ohaus Pioneer Analytical Balances. Both adult male frogs and adult female frogs were separated into each sampling period (i.e., July 2020, October 2020, and February 2021).

5. Statistical analysis

All parameters were priorly tested for normality and homogeneity of variance. For gonad, liver, and body weights, rice frogs were analyzed separately between adult male and adult female for each site. For liver weight and body weight, male and female data were pooled in the absence of a significant sex-related difference. Differences in gonad and liver weights between sites were carried out by analysis of covariance (ANCOVA), by controlling the influence of body weight (body weight as a covariable) followed by Bonferroni tests and power of the tests analysis. Differences in body weight between sites was carried out by analysis of covariance (ANCOVA) by controlling the influence of snout-vent length (snout-vent length as a covariable) followed by Bonferroni tests and power of the tests analysis. All ANCOVA analyses were expressed as estimated marginal means, the mean response for each factor, adjusted for any other variables in the model. Statistical analysis was done using SPSS Stat 28 for MacOS.

Results

1. Gonad weight

1.1. Testicular weight

After controlling the influence of body weight, the statistical analysis showed that there was no significant site-related difference in testicular weight during July 2020, October 2020, and February 2021 (Table 4.1, Figure 4.1).

Table 4.1 ANCOVA analysis of testicular weight of adult male frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Period (*n* Contaminated: Reference)	**Contaminated site** (Mean ± SEM)	**Reference site** (Mean ± SEM)	**Statistical value**
July 2020 (20: 35)[a] Late wet season	0.015 ± 0.001	0.014 ± 0.001[ns]	$F_{1,52}$ = 0.075, p = 0.785, Power of test (α = 0.05) = 0.058
October 2020 (6: 4)[a] Early dry season	0.002 ± 0.001	0.005 ± 0.001[ns]	$F_{1,7}$ = 1.499, p = 0.260, Power of test (α = 0.05) = 0.186
February 2021 (10: 11)[a] Late dry season	0.012 ± 0.001	0.011 ± 0.001[ns]	$F_{1,18}$ = 0.175, p = 0.680, Power of test (α = 0.05) = 0.068

Remarks:

[a] Compared by ANCOVA and expressed as estimated marginal means adjusted for body weight

[ns] No significant difference between sites; $p > 0.05$

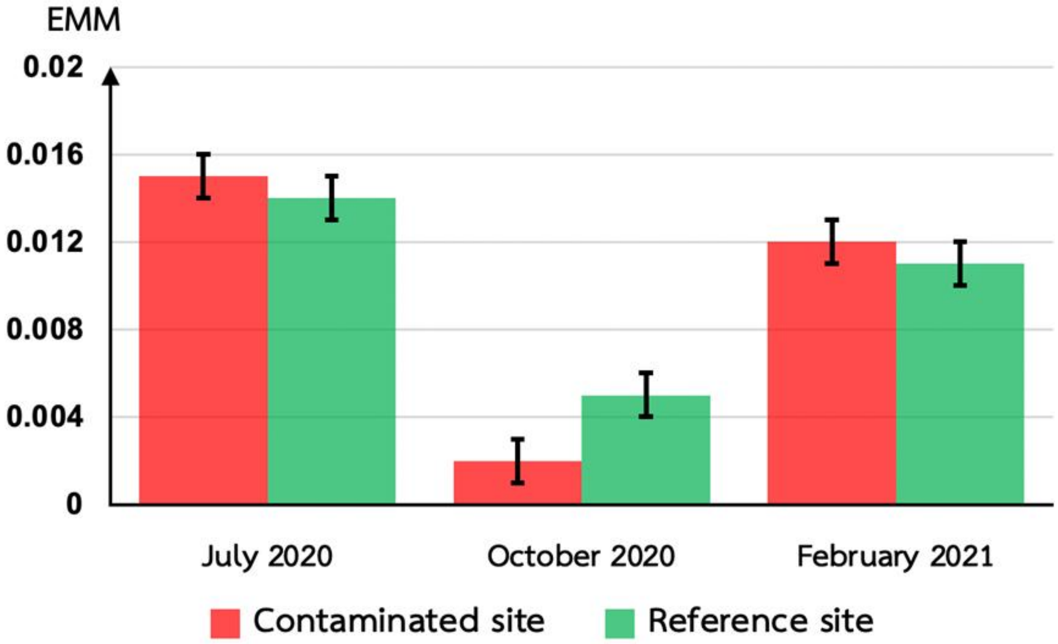

Figure 4.1 ANCOVA analysis of testicular weight (Mean ± SEM) of adult male frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021. Abbreviations: (EMM) estimated marginal means

1.2. Ovarian weight

After controlling the influence of body weight, the statistical analysis showed that there was no significant site-related difference in ovarian weight during July 2020, October 2020, and February 2021. There was an observably higher estimated marginal means of ovarian weight from contaminated site during July 2020 period, although not significantly (Table 4.2, Figure 4.2).

Table 4.2 ANCOVA analysis of ovarian weight of adult female frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Period (*n* Contaminated: Reference)	**Contaminated site** (Mean ± SEM)	**Reference site** (Mean ± SEM)	**Statistical value**
July 2020 (28: 14)[a] Late wet season	0.879 ± 0.052	0.681 ± 0.082[ns]	$F_{1,39}$ = 3.234, p = 0.080, Power of test (α = 0.05) = 0.419
October 2020 (2: 2)[a] Early dry season	0.067 ± 0.003	0.072 ± 0.003[ns]	$F_{1,1}$ = 1.246, p = 0.465, Power of test (α = 0.05) = 0.078
February 2021 (10: 10)[a] Late dry season	0.443 ± 0.123	0.547 ± 0.123[ns]	$F_{1,17}$ = 0.355, p = 0.559, Power of test (α = 0.05) = 0.087

Remarks:

[a] Compared by ANCOVA and expressed as estimated marginal means adjusted for body weight

[ns] No significant difference between sites; $p > 0.05$

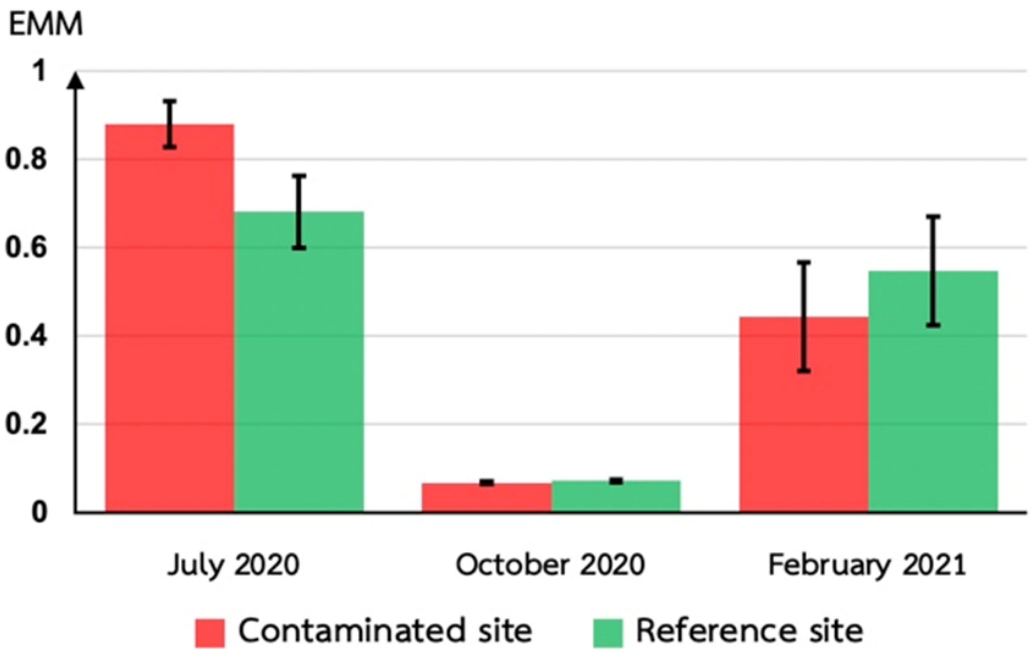

Figure 4.2 ANCOVA analysis of ovarian weight (Mean ± SEM) of adult female frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021. Abbreviations: (EMM) estimated marginal means

2. Liver weight

After controlling the influence of body weight, the statistical analysis showed that there was no significant sex-related difference in liver weight from contaminated site and reference site throughout sampling periods (Appendix E). Therefore, the male and female data were pooled. The statistical analysis showed that there was significant site-related difference in liver weight of adult rice frogs during February 2021. There was no significant site-related difference in liver weight of adult rice frogs during July and October 2020. (Table 4.3, Figure 4.3).

Table 4.3 ANCOVA analysis of liver weight of adult rice frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Period (*n* Contaminated: Reference)	Contaminated site (Mean ± SEM)	Reference site (Mean ± SEM)	Statistical value
July 2020 (48: 49)[a] Late wet season	0.177 ± 0.005	0.166 ± 0.005[ns]	$F_{1,94}$ = 1.719, p = 0.193, Power of test (α = 0.05) = 0.254
October 2020 (8: 6)[a] Early dry season	0.122 ± 0.013	0.136 ± 0.016[ns]	$F_{1,11}$ = 0.418, p = 0.531, Power of test (α = 0.05) = 0.091
February 2021 (20: 21)[a] Late dry season	0.096 ± 0.005	0.115 ± 0.005[*]	$F_{1,38}$ = 7.214, p = 0.011, Power of test (α = 0.05) = 0.745

Remarks:

[a] Compared by ANCOVA and expressed as estimated marginal means adjusted for body weight

[*] Significant difference between sites; $p \leq 0.05$

[ns] No significant difference between sites; $p > 0.05$

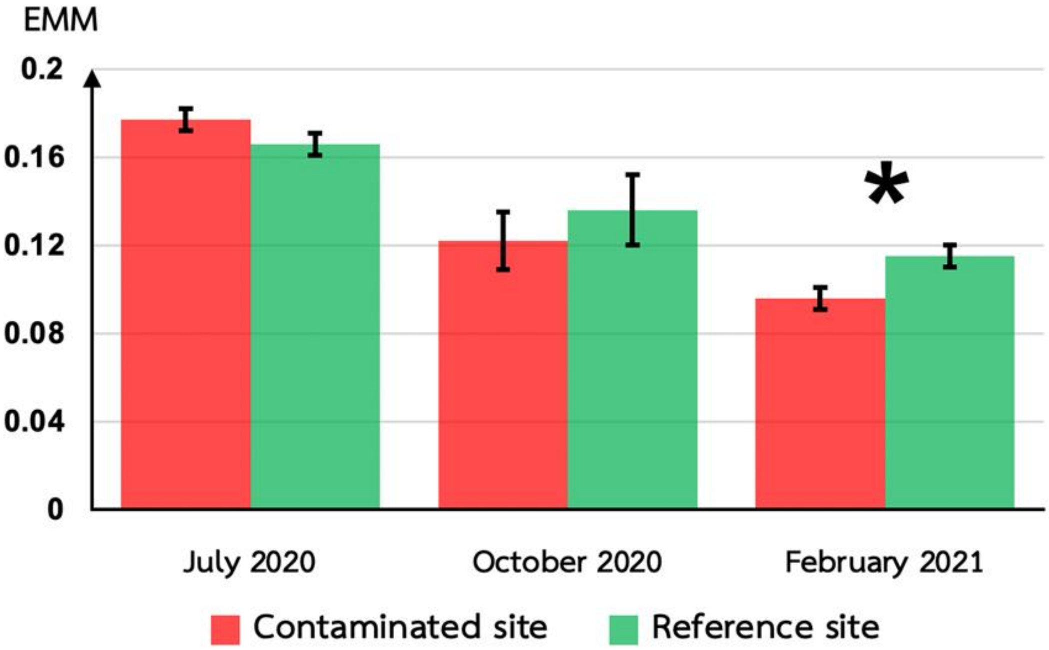

Figure 4.3 ANCOVA analysis of liver weight (Mean ± SEM) of adult rice frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021. Abbreviations: (EMM) estimated marginal means

3. Body weight

After controlling the influence of snout-vent length, the statistical analysis showed that there was no significant sex-related difference in body weight from contaminated site and reference site throughout sampling periods (Appendix E). Therefore, the male and female data were pooled. The statistical analysis showed that there was significant site-related difference in body weight of adult rice frogs during July 2020. There was no significant site-related difference in body weight of adult rice frogs during October 2020 and February 2021 (Table 4.4, Figure 4.4).

Table 4.4 ANCOVA analysis of body weight of adult rice frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Period (*n* Contaminated: Reference)	Contaminated site (Mean ± SEM)	Reference site (Mean ± SEM)	Statistical value
July 2020 (54: 102)[a] Late wet season	6.971 ± 0.182	6.382 ± 0.123[*]	$F_{1,153}$ = 5.898, p = 0.016, Power of test (α = 0.05) = 0.675
October 2020 (14: 12)[a] Early dry season	7.212 ± 0.227	7.390 ± 0.245[ns]	$F_{1,23}$ = 0.281, p = 0.601, Power of test (α = 0.05) = 0.080
February 2021 (29: 64)[a] Late dry season	5.596 ± 0.217	5.750 ± 0.145[ns]	$F_{1,90}$ = 0.345, p = 0558, Power of test (α = 0.05) = 0.090

Remarks:

[a] Compared by ANCOVA and expressed as estimated marginal means adjusted for snout-vent length

[*] Significant difference between sites; $p \leq 0.05$

[ns] No significant difference between sites; $p > 0.05$

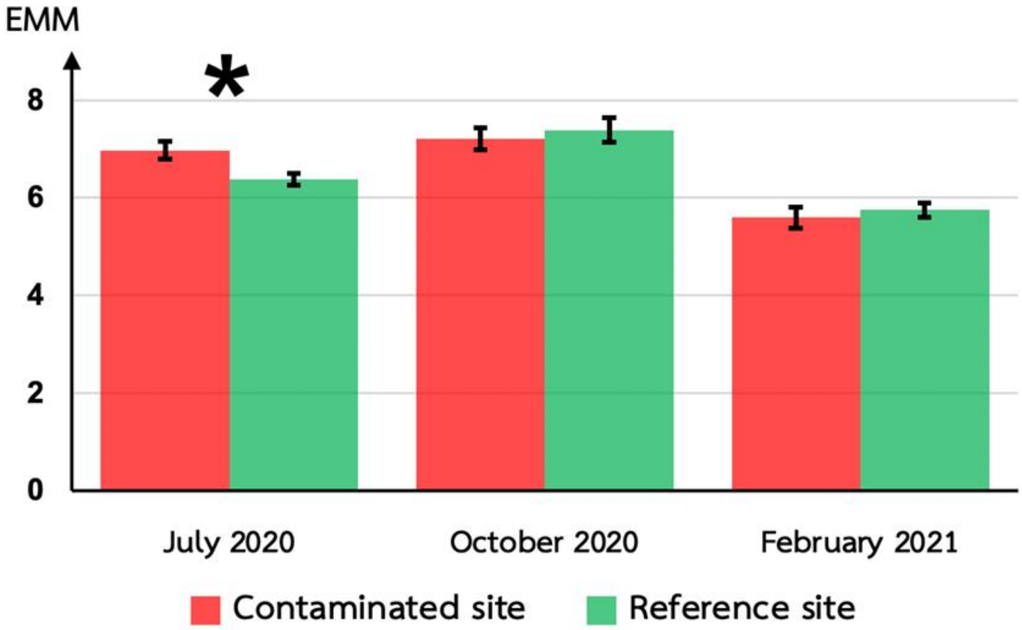

Figure 4.4 ANCOVA analysis of body weight (Mean ± SEM) of adult rice frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021. Abbreviations: (EMM) estimated marginal means

Discussions

Previous research utilizing the somatic index indicated that morphological alterations occurred in the gonad and liver (Thammachoti, 2012). However, such a direct approach (somatic index = organ weight x 100/ body weight) may not account for frog's ontogeny factor since growth patterns may vary at different stages of life. To overcome these limitations, analysis of covariance (ANCOVA) may resolve this issue by taking into account the frogs' growth patterns, as ANCOVA analysis has been previously used to monitor the adverse effect on non-target organisms such as turtles (Rie et al., 2005; Kitana et al., 2007)

The morphological alterations in *F. limnocharis* were evidenced, based on the results of several organismal parameters. Although there was no significant site-related difference for both testicular and ovarian weights throughout periods (Tables 4.1–4.2, Figures 4.1–4.2)—which may be due to high individual variation and small sample size of adult frogs, there was an observably higher estimated marginal means of ovarian weight from contaminated site during July 2020, which are similar to the prior studies (Thammachoti, 2012). Previous studies using relative gonad weight (gonadosomatic index) showed that there was significantly heavier ovarian weight from contaminated site, especially during late wet season (Thammachoti, 2012). The larger ovary is possibly due to the effects of xenoestrogen on ovarian growth. Atrazine is a known endocrine-disrupting chemical (Hayes et al., 2006; Fan et al., 2007) that can exert its adverse effect, e.g., complete feminization and chemical castration in male *Xenopus laevis* (Hayes et al., 2010), hermaphroditism (Hayes et al., 2002), and shifted sex ratios in *Acris blanchardi* (Hoskins and Boone, 2018).

The results indicated a gradual decrease of gonad weight toward the early dry season (October 2020). It has been known that *F. limnocharis* is a seasonal breeder, having cyclic reproduction mode synchronized with rainfall (Othman et al., 2011), where two main waves of increases female gonadosomatic index occur just before the beginning of rainy season and the end of rainy season, also, gonadosomatic index would be significantly lower at dry season. However, previous investigation in contaminated site revealed that gravid female, containing fully-matured eggs, was found even in dry period, which is not beneficial to the frogs since they cannot lay their eggs in dried-up water bodies (Thammachoti, 2012). Consequently, the effect of atrazine or other herbicides may lead to reduce amphibian fecundity living in contaminated site, as it was evidenced in the current study.

The results on liver weight of adult frogs (Table 4.3) showed that there was a significant site-related difference during February 2021, where higher liver weight was observed from reference site. This may be due to the liver being used as the detoxification organ (Crawshaw and Weinkle, 2000), as it was reported that paraquat can reduce glutathione levels in liver of *Channa punctata,* thereby, the liver must work extensively to eliminate these contaminants (Parvez and Raisuddin, 2006). The possible explanation could be that xenobiotics induce reactive oxygen species formation (Hansen et al., 2006) which may lead to an increase in lipid peroxidation (Isani et al., 2009), resulting in cell death via apoptosis and/ or necrosis (Norris et al., 2000). These have been observed in the previous studies from Tak province (Othman et al., 2016) showing a lower hepatosomatic index from the cadmium-contaminated site. The histopathological examination would address these discrepancies and further confirm the influence of herbicide on the liver of rice frogs.

Similar to the trend of gonad weight throughout periods, the gradual increase of liver weight in late wet season (July 2020) on adult frogs may be synchronized with the rainfall and peak herbicide utilization during that period. Since herbicide contamination in tissue of rice frog during early dry season (February 2021) was evidenced in this study (Chapter III), it cannot be ruled out that the contamination would be much higher during peak herbicide utilization (late wet season).

The results on body weight of adult frogs (Table 4.4) showed that there was a significant site-related difference in July 2020, where higher body weight was observed from contaminated site. These may be partly contributed by gonad and liver that showed an observably higher ovarian weight and liver weight, although not significantly (Tables 4.2–4.3, Figures 4.2–4.3). It is believed that the difference in body weight was influenced by environmental stressors (Söderman et al., 2007; Thammachoti, 2012) potentially caused by paraquat (Dial and Bauer, 1984; Vismara et al., 2000; Osano et al., 2002) and/ or glyphosate (Bach et al., 2016; Babalola et al., 2019), which may lead to disruption on steroidogenesis and growth hormone secretion (Hayes et al., 2006). The other possible explanation is simply due to the aestivation period when their food sources were unavailable (Hirai and Matsui, 2001). Nonetheless, previous studies by Thammachoti et al. (2012) revealed a lower condition factor (indicator of overall health) on adult frogs from contaminated site, meaning that if the SVL of frogs is equal, the lighter frogs may have lesser health fitness (Boone, 2005), which is not the case with the results of the current study.

Of interest with regard to the reference site was a large number of froglet–juvenile and adult frogs collected compared to the contaminated site (Appendix D). These may be due to the differences in population structure in these two sites. Information on age structure would allow one to determine if there is any difference in maturation from the contaminated and reference sites.

Conclusion

There was an observably higher estimated marginal means of ovarian weight from the contaminated site during July 2020, suggesting a negative impact of xenoestrogen exposure. The significant site-related difference in liver weight might be due to xenobiotic exposure. Therefore, when and if these two organismal approaches are used to monitor the rice frog's health status, the numbers of samples and seasonal period should be considered. The significant site-related difference in body weight of rice frogs during July 2020 may be contributed by liver and ovarian weights. Based on organismal parameters, it can be suggested that concurrent and intensive utilization of herbicides in paddy fields may induce morphological alterations in rice frogs. Further analysis for an impact of these contaminations on the rice frogs based on population parameters is recommended.

CHAPTER V

HEALTH STATUS OF RICE FROGS *Fejervarya limnocharis* POPULATIONS LIVING IN AGRICULTURAL AREAS OF NAN PROVINCE, THAILAND BASED ON POPULATION PARAMETERS

Introduction

Nan Province, located on the northern side of Thailand, is known as an area with high agricultural activities. In this region, herbicides were used intensively and continuously (Chanpong, 2008), resulting in contamination by several herbicides, i.e., glyphosate and atrazine, in paddy field environment (Thammachoti, 2012; Jantawongsri et al., 2015). In addition, it has been confirmed that herbicides residues were present in non-target organism tissues (Chapter III). Therefore, this agrochemical contamination may ultimately become an environmental stressor for their ecosystem.

Sentinel species may reflect the adverse effect of environmental stressors from agrochemical contamination in the population since it shares similar features of their organs to humans (Venturino et al., 2003). Then, it is crucial to monitor the extent of herbicide contamination on sentinel species populations to provide an early warning of health risks and environmental hazards caused by agrochemical contamination.

Amphibians have been regarded as excellent sentinel species for environmental change or stressors induced by herbicide contamination (Thammachoti, 2012). Since they lived both in terrestrial and aquatic habitats, they are most likely susceptible and sensitive to environmental stress (Venturino et al., 2003), especially through their semi-permeable skin (Roy, 2002).

The global decline of amphibians has been linked to environmental pollution (Mann et al., 2009). The unfavorable conditions and increased environmental stressors may greatly affect their growth and survival in the population (Gahl et al., 2011). Previous studies have reported differences in growth pattern of amphibians living in areas with different degrees of agrochemical contamination (Thammachoti et al., 2012; Hegde and Krishnamurthy, 2014). Moreover, agrochemical contamination may lead to disruption on population structure—an indication of the reproductive capabilities and probability of the continuation of species, as previously reported that shift in sex ratios may be associated with endocrine-disrupting chemicals (EDCs) (Lambert et al., 2015). In addition, agrochemical contamination may lead to an increase of fluctuating asymmetry (FA)—organisms' deviation from ideal bilateral symmetry traits (Palmer and Strobeck, 1986). It is believed that environmental stressor has contributed to higher developmental instability by showing a higher FA, as previously reported on various amphibians' species (Lauck, 2006; Söderman et al., 2007; Thammachoti, 2012; Zhelev et al., 2015a; Costa and Nomura, 2016; Eterovick et al., 2016; Zhelev et al., 2019). Therefore, these parameters on population levels could be used to monitor the impact of continuous and intensive herbicide utilization on amphibian population.

In the last segment of this study, the influence of herbicides utilization was investigated on rice frogs *Fejervarya limnocharis* which can be found abundantly in paddy fields, making it susceptible to prolong exposure to herbicides. The health status of rice frogs living in paddy fields with different degrees of herbicides utilization was examined based on the population parameters, i.e., growth pattern, size-frequency distribution, and fluctuating asymmetry.

Hypothesis

There are significant differences in growth pattern, fluctuating asymmetry, and size-frequency distribution between rice frogs *F. limnocharis* living in contaminated agricultural area with those living in reference agricultural area.

Objective

To examine health of rice frogs *F. limnocharis* populations living in agricultural areas of Nan Province, Thailand based on population parameters

Materials and Methods

1. Study sites

The study sites are in Wiang Sa District, Nan Province, Thailand. In this region, there are many agricultural areas where herbicides have been utilized for a long time. The potentially contaminated site is a paddy field with intensive herbicide utilization (Yupin Chairaja, personal communication, October 6, 2009) (San Sub-district; 18°30’37.9297” to 18°30’25.6926”; 100°46’24.2638” to 100°46’04.9957”), while the reference site is a paddy field with no history of herbicide utilization for more than 10 years (Srinoon Kamsrikaew, personal communication, October 6, 2009) (Lai-Nan Sub-district; 18°34’35.4157” 18°34’24.5818”; 100°46’27.5499” to 100°46’12.0212”). The reference site practiced one crop cycle by planting a single crop in the same field throughout the growing season, whereas the contaminated site practiced two-crop cycle by planting two or more crops in the same field throughout the growing season, with corn and rice being the most frequently planted crops (Appendix A).

2. Sampling periods

Samplings were conducted during July and October 2020, and February 2021 covering the seasonal period of wet–dry seasons (Appendix B) and agricultural cultivation cycle (Appendix A). Life cycles of rice frogs includes several stages, including egg, tadpole, froglet, juvenile, sub-adult, and adult (Duellman and Trueb, 1994), where abundances may vary according on season and water availability (Appendix B, Appendix D). Field samplings were conducted using purposive sampling method by visual encounter survey (Kusrini, 2019) where several stages of frogs (i.e., froglet, juvenile, sub-adult, adult) were caught by hand at night considering the active time of the species. To get the best comparable data, the survey was limited to 4–6 surveyors and restricted to 45 minutes per site. The total sample of rice frogs collected from contaminated and reference sites is shown in Appendix D.

3. Sample collection

The animal care and use protocol in this study has been approved by the institutional animal care and use committee of the Faculty of Science, Chulalongkorn University (CU-ACUP No. 2123002). Upon arrival in the laboratory at Chulalongkorn University Forest and Research Station, Wiang Sa district, Nan Province, frogs were immediately euthanized by immersion in 0.5% tricaine methane sulfonate solution (Sigma-Aldrich, St. Louis, MO, USA). The juvenile, sub-adult, and adult frogs were dissected to further confirms the sex of each individual with the aid of a stereomicroscope (Carl Zeiss).

The appendage bones were used for the fluctuating asymmetry analysis. The carcass of each individual was boiled ad 100°C for 10–20 minutes. The tissue was removed using forceps and a small bristle brush leaving only the skeleton of the frogs. Subsequently, 2 appendage bones of forelimb (i.e., radio-ulna, humerus) and 3 appendage bones of hindlimb (i.e., femur, tibio-fibula, astragalus-calcaneum) (Figure 5.1) were individually and separately collected from the left and right side of each individual and marked. These bones were kept in a hot air oven at 60°C for 24 hours, until completely dry. The bone-dry samples were individually kept in small plastic containers with desiccant (Figure 5.2), placed in the covered storage to avoid sunlight, and kept at room temperature (25–35°C) until further analysis.

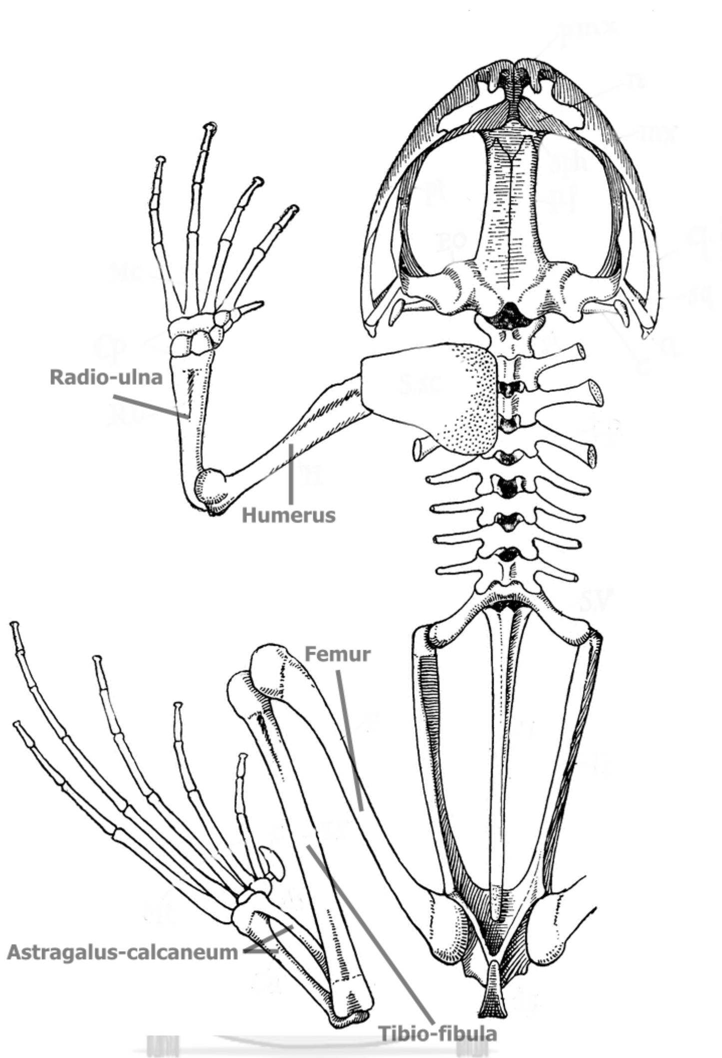

Figure 5.1 Appendage bones of frogs including forelimb (radio-ulna, humerus) and hindlimb (femur, tibio-fibula, astragalus-calcaneum) used for fluctuating asymmetry analysis in this study (University of South Florida, 2021).

Figure 5.2 Bone-dry samples were individually kept in small plastic containers.

4. Estimation on growth pattern

The samples were measured for body weight (BW) (g) and snout-vent length (SVL) (mm) using Ohaus Pioneer Analytical Balances (accuracy 0.0001 g) and Mitutoyo Absolute Digimatic Caliper (accuracy 0.01 mm), respectively. For the growth pattern, regression analysis was calculated based on log-transformed data of body weight and snout-vent length of overall data represented in equation (1),

$$\log BW = b\log SVL + \log a \ldots (1)$$

where constant b is a scaling coefficient—indicating the growth pattern of the population (Othman, 2009).

5. Estimation on size-frequency distribution

For the size-frequency distribution, the dissected frogs were determined for stage and sex then categorized into froglet, male, and female. Additional samples collected beyond the primary sampling from reference site (Appendix D), were omitted from the analysis (n = 31) to achieve representative results under similar effort. The frogs were categorized by snout-vent length (SVL) (mm) and compared as whole population (including froglet, male, and female) and separately by sex (male and female). The data from each site were represented in relative frequency in percentage using base in R v.3.4.1 (R Core Team, 2017).

6. Estimation on fluctuating asymmetry

The bone-dry sample was measured for length with Moore and Wright micrometer (accuracy 0.001 mm) and weight using Ohaus Pioneer Analytical Balances (accuracy 0.0001 g). The measurement for length and weight was repeated two times to avoid bias in measurement.

7. Statistical analysis

For growth pattern data of overall populations, general linear model was used for analysis, including power of the tests. Differences in size-frequency distribution of overall populations were compared by two-sample Kolmogorov-Smirnov test.

For the fluctuating asymmetry, the analyses were separated between weight and length, between sex, for each appendage bone. A step-by-step flow chart modified from Thammachoti (2012) was followed (Figure 5.3). To investigate the fluctuating asymmetry in each population, three factors contributing to trait difference must be priorly estimated (Palmer, 1994), i.e., i) side variation as directional asymmetry (DA), ii) individual variation as size/ shape variation, and iii) interaction between side variation and individual variation as non-directional asymmetry/ fluctuating asymmetry. The significance ($p \leq 0.05$) of these three factors was analyzed using two-way analysis of variance (ANOVA) by using side variation (left and right) as the first factor, individual variation as the second factor, and bone weight and bone length as the dependent variables. Typically, two-way ANOVA tests are two-tailed, however, FA analysis should include a priori assumptions about any direction of relationships, which is referred to as one-tailed testing, where the assumption is appropriate if the estimated value departs only in one way from the reference value (which is site-related difference on FA). By disregarding null hypothesis, the current study hypothesized that there exist site-related differences in FA.

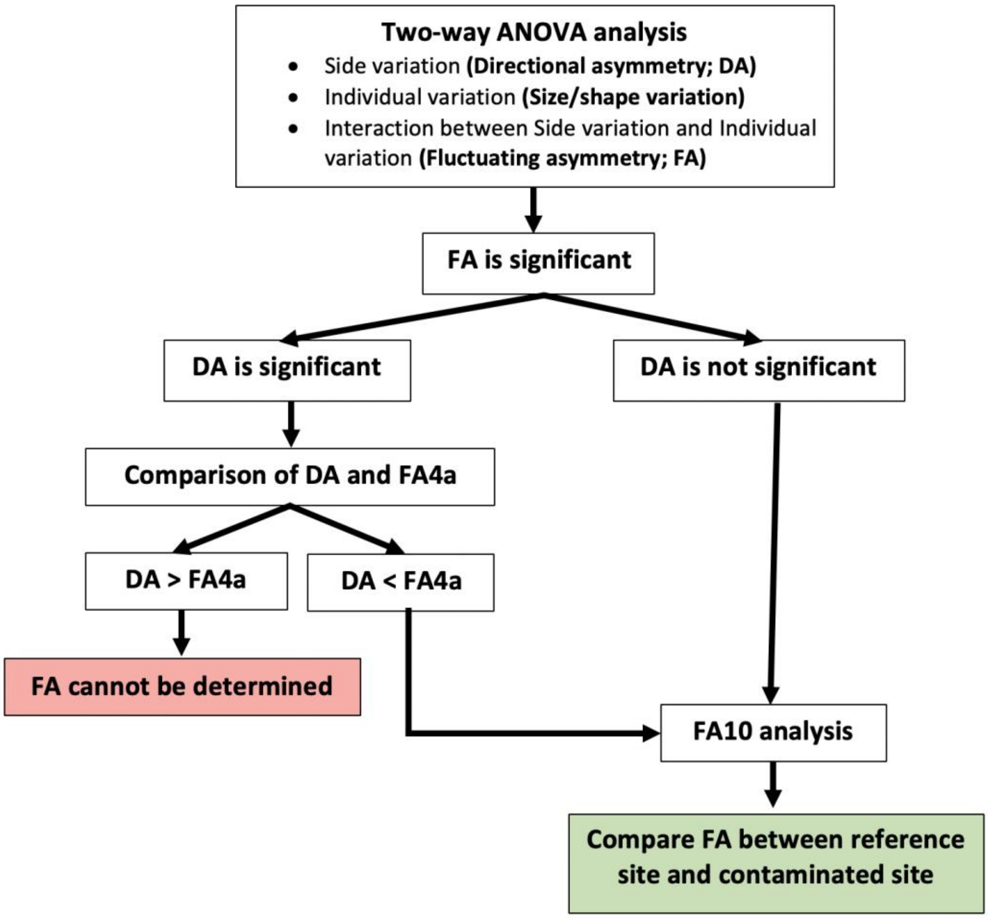

Figure 5.3 Step-by-step flowchart of fluctuating asymmetry analysis (Palmer, 1994; Palmer and Strobeck, 2003; Thammachoti, 2012)

In case two-way ANOVA indicates that DA is significant, additional analysis is needed to confirm that DA was not interfering with FA. Otherwise, the trait with significant DA cannot be determined for FA (Palmer, 1994). The interference was investigated by comparing the DA and FA4a of each population. The DA index of each population was calculated using the following equation (Palmer and Strobeck, 2003) (2),

Directional asymmetry (DA) = mean (R – L)...(2)

where the mean value of difference between right side (R) and left side (L) was calculated. Moreover, the FA4a index of each population was calculated using the following formula (Palmer and Strobeck, 2003) (3),

FA4a = 0.798 × $\sqrt{variance\ R - L}$...(3)

If DA is less than FA4a, it means that DA does not interfere with FA interpretation, hence, the data were subjected to further FA10 index. However, if the DA is higher than FA4a, it means that DA is probably interfering with FA interpretation, and by inference, the DA was significant that FA cannot be determined. Upon meeting this requirement, FA10 analysis of each population was calculated using the following formula (Palmer, 1994) (4),

FA10 = (mean square interaction [sid. x ind.] – mean square of residual)/ 2...(4)

and to tests for significant site-related difference, analysis of Fisher-Snedecor distribution was performed by using the degree of freedom from FA10 analysis results (Palmer, 1994). All statistical analyses were carried out by SPSS Stat 28 for MacOS and R v.3.4.1 (R Core Team, 2017).

Results

1. Growth pattern

Growth patterns of rice frog *F. limnocharis* from contaminated site and reference site were shown as weight-length relationship curves. The scaling coefficient (slope of the line) of frogs from contaminated site (2.8371) was higher than those in reference site (2.6569). Moreover, comparison of the growth pattern carried out by the general linear model statistical analysis showed that there was a significant site-related difference ($F_{1,377}$ = 4.838, p = 0.028, Power of test [α = 0.05] = 0.593) (Figure 5.4).

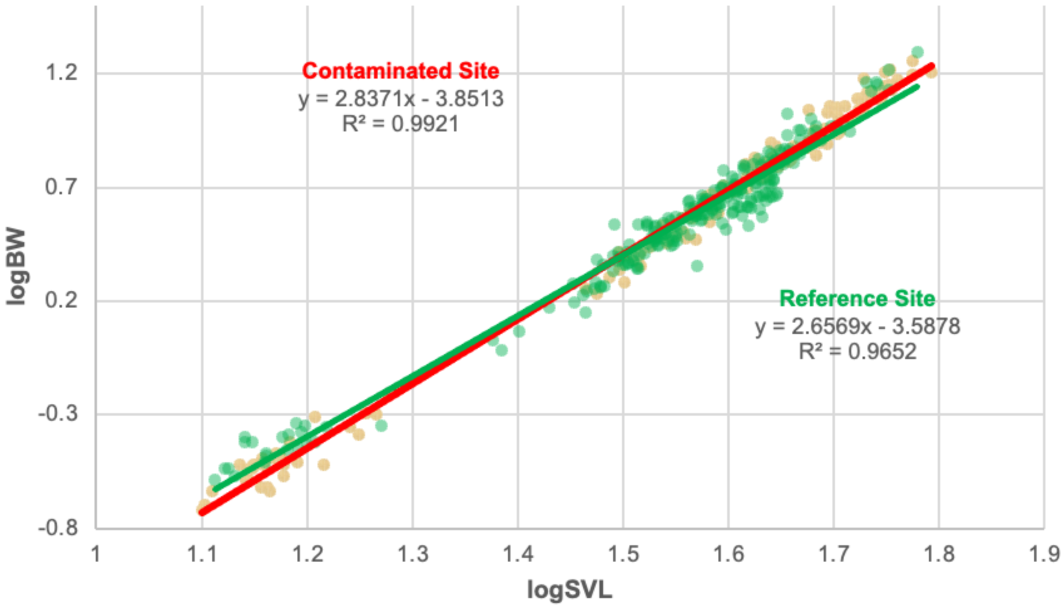

Figure 5.4 Weight-length relationship curves of rice frog *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

2. Fluctuating asymmetry

2.1. Adult male bone length

2.1.1. Two-way ANOVA to test for FA assumption

Statistical analysis showed that side variation on length of 5 appendage bones was not significant in both sites, suggesting that directional asymmetry (DA) was not present. The individual variation was significant in both sites, meaning that size/shape variation was present. Lastly, the interaction between side and individual variations suggests a presence of FA (Table 5.1). Thereby, the FA on length of 5 appendage bones of adult male frogs can be determined.

Table 5.1 Two-way ANOVA on bone length of adult male frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Male bone length	Contaminated site		Reference site	
(*n* Contaminated: Reference)	Mean square	*p*-value	Mean square	*p*-value
Radio-ulna (29: 76)				
Side variation	0.19×10^{-2}	> 0.05	0.43×10^{-2}	> 0.05
Individual variation	2.18	≤ 0.05	1.36	≤ 0.05
Interaction (sid. x ind.)	2.45×10^{-2}	≤ 0.05	6.99×10^{-2}	≤ 0.05
residual	5.70×10^{-4}	–	1.20×10^{-4}	–
Humerus (29: 72)				
Side variation	5.68×10^{-2}	> 0.05	0.37×10^{-2}	> 0.05
Individual variation	4.92	≤ 0.05	4.54	≤ 0.05
Interaction (sid. x ind.)	5.02×10^{-2}	≤ 0.05	6.87×10^{-2}	≤ 0.05
residual	3.00×10^{-4}	–	1.00×10^{-4}	–
Femur (29: 72)				
Side variation	28.92×10^{-2}	> 0.05	0.70×10^{-2}	> 0.05
Individual variation	10.62	≤ 0.05	9.07	≤ 0.05
Interaction (sid. x ind.)	14.50×10^{-2}	≤ 0.05	2.66×10^{-2}	≤ 0.05
residual	1.00×10^{-4}	–	0	–
Tibio-fibula (29: 73)				
Side variation	2.33×10^{-2}	> 0.05	11.22×10^{-2}	> 0.05
Individual variation	15.34	≤ 0.05	13.09	≤ 0.05
Interaction (sid. x ind.)	5.49×10^{-2}	≤ 0.05	6.04×10^{-2}	≤ 0.05
residual	3.00×10^{-4}	–	0	–
Astragalus-calcaneum (29: 73)				
Side variation	11.01×10^{-2}	> 0.05	1.32×10^{-2}	> 0.05
Individual variation	4.42	≤ 0.05	4.13	≤ 0.05
Interaction (sid. x ind.)	3.97×10^{-2}	≤ 0.05	2.07×10^{-2}	≤ 0.05
residual	3.00×10^{-4}	–	1.00×10^{-4}	–

2.1.2. Comparison of FA in male bone length between sites

Statistical analysis by comparing FA10 between sites using Fisher-Snedecor distribution revealed that there were significant site-related differences of FA in femur and astragalus-calcaneum lengths of adult male frogs, showing higher FA from contaminated site. However, there was no significant site-related difference of FA in radio-ulna, humerus, and tibio-fibula lengths of adult male frogs (Table 5.2, Figure 5.5).

Table 5.2 Fluctuating asymmetry of bone length of adult male frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Male bone length	**Contaminated site**		**Reference site**		**Statistical**
(*n* Contaminated: Reference)	df	FA	df	FA	**value**
Radio-ulna (*n* 29: 76)	26.70	1.20×10^{-2}	74.75	3.49×10^{-2}	$p > 0.05$
Humerus (*n* 29: 72)	27.66	2.45×10^{-2}	70.80	3.43×10^{-2}	$p > 0.05$
Femur (*n* 29: 72)	27.94	7.24×10^{-2}	71.99	1.32×10^{-2}	$p \leq 0.05$
Tibio-fibula (*n* 29: 73)	27.67	2.72×10^{-2}	71.90	3.01×10^{-2}	$p > 0.05$
Astragalus-calcaneum (*n* 29: 73)	27.60	1.97×10^{-2}	71.54	1.03×10^{-2}	$p \leq 0.05$

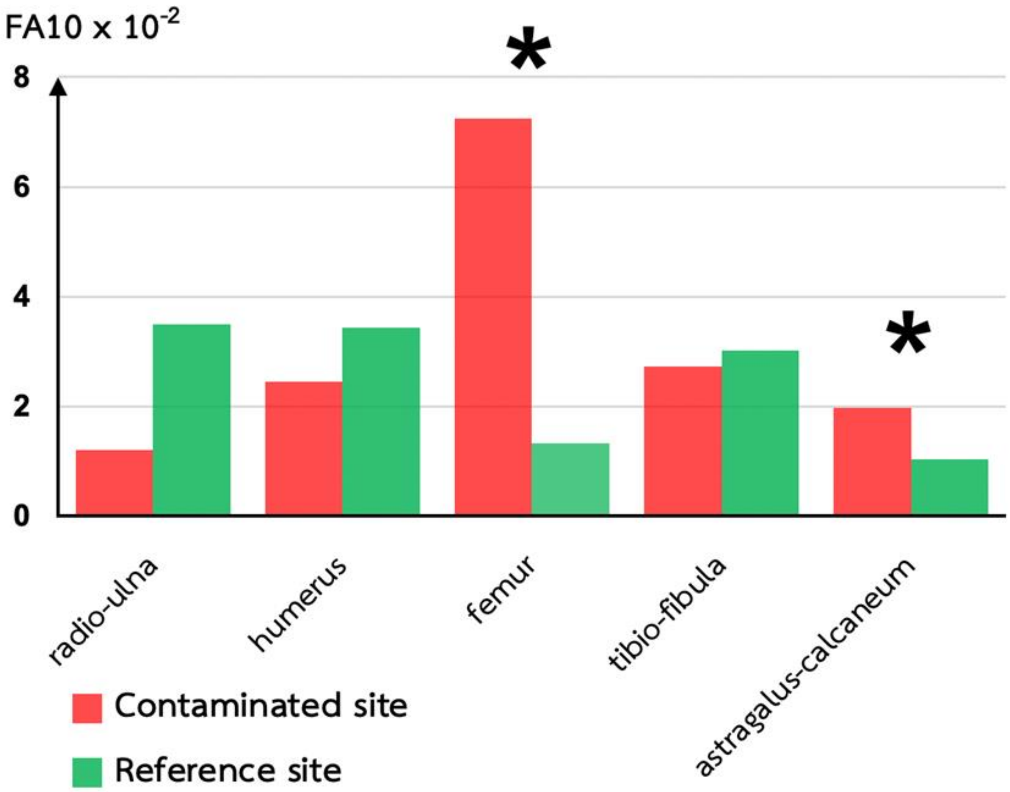

Figure 5.5 Fluctuating asymmetry of bone length of adult male frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021. Asterisk (*) indicates significant difference between sites, $p \leq 0.05$

2.2. Adult male bone weight

2.2.1. Two-way ANOVA to test for FA assumption

Statistical analysis showed that side variation on weight of 5 appendage bones was not significant in both sites, suggesting that directional asymmetry (DA) was not present. The individual variation was significant in both sites, meaning that size/shape variation was present. Lastly, the interaction between side and individual variations suggests a presence of FA (Table 5.3). Thereby, the FA on weight of 5 appendage bones of adult male frogs can be determined.

Table 5.3 Two-way ANOVA on bone weight of adult male frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Male bone weight	Contaminated site		Reference site	
(*n* Contaminated: Reference)	Mean square	*p*-value	Mean square	*p*-value
Radio-ulna (29: 75)				
Side variation	4.20×10^{-9}	> 0.05	2.58×10^{-7}	> 0.05
Individual variation	1.76×10^{-5}	≤ 0.05	4.17×10^{-6}	≤ 0.05
Interaction (sid. x ind.)	7.94×10^{-8}	≤ 0.05	1.39×10^{-7}	≤ 0.05
residual	1.32×10^{-8}	–	1.34×10^{-8}	–
Humerus (29: 71)				
Side variation	3.10×10^{-7}	> 0.05	2.48×10^{-7}	> 0.05
Individual variation	7.57×10^{-5}	≤ 0.05	2.08×10^{-5}	≤ 0.05
Interaction (sid. x ind.)	1.76×10^{-7}	≤ 0.05	2.05×10^{-7}	≤ 0.05
residual	8.00×10^{-9}	–	17.50×10^{-8}	–
Femur (29: 73)				
Side variation	1.57×10^{-6}	> 0.05	0.29×10^{-6}	> 0.05
Individual variation	1.99×10^{-4}	≤ 0.05	0.62×10^{-4}	≤ 0.05
Interaction (sid. x ind.)	1.67×10^{-6}	≤ 0.05	0.27×10^{-7}	≤ 0.05
residual	1.00×10^{-8}	–	1.80×10^{-8}	–
Tibio-fibula (29: 73)				
Side variation	1.46×10^{-6}	> 0.05	1.07×10^{-6}	> 0.05
Individual variation	4.64×10^{-4}	≤ 0.05	1.26×10^{-4}	≤ 0.05
Interaction (sid. x ind.)	1.67×10^{-5}	≤ 0.05	0.07×10^{-5}	≤ 0.05
residual	1.00×10^{-8}	–	1.90×10^{-8}	–
Astragalus-calcaneum (29: 71)				
Side variation	6.30×10^{-8}	> 0.05	1.27×10^{-6}	> 0.05
Individual variation	7.61×10^{-5}	≤ 0.05	2.26×10^{-5}	≤ 0.05
Interaction (sid. x ind.)	5.90×10^{-7}	≤ 0.05	2.32×10^{-7}	≤ 0.05
residual	1.40×10^{-8}	–	1.33×10^{-8}	–

2.2.2. Comparison of FA in male bone weight between sites

Statistical analysis by comparing FA10 between sites using Fisher-Snedecor distribution revealed that there were significant site-related differences of FA in femur, tibio-fibula, and astragalus-calcaneum weights of adult male frogs, showing higher FA from contaminated site. However, there was no significant site-related difference of FA in radio-ulna, and humerus of adult male frogs (Table 5.4, Figure 5.6).

Table 5.4 Fluctuating asymmetry of bone weight of adult male frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Male bone weight	Contaminated site		Reference site		Statistical
(*n* Contaminated: Reference)	df	FA	df	FA	value
Radio-ulna (*n* 29: 75)	19.21	0.33×10^{-7}	60.13	0.62×10^{-7}	$p > 0.05$
Humerus (*n* 29: 71)	25.45	0.84×10^{-7}	58.35	9.39×10^{-7}	$p > 0.05$
Femur (*n* 29: 73)	27.66	8.31×10^{-7}	62.52	1.26×10^{-7}	$p \leq 0.05$
Tibio-fibula (*n* 29: 73)	27.96	83.40×10^{-7}	68.40	3.75×10^{-7}	$p \leq 0.05$
Astragalus-calcaneum (*n* 29: 71)	27.67	2.88×10^{-7}	62.14	1.09×10^{-7}	$p \leq 0.05$

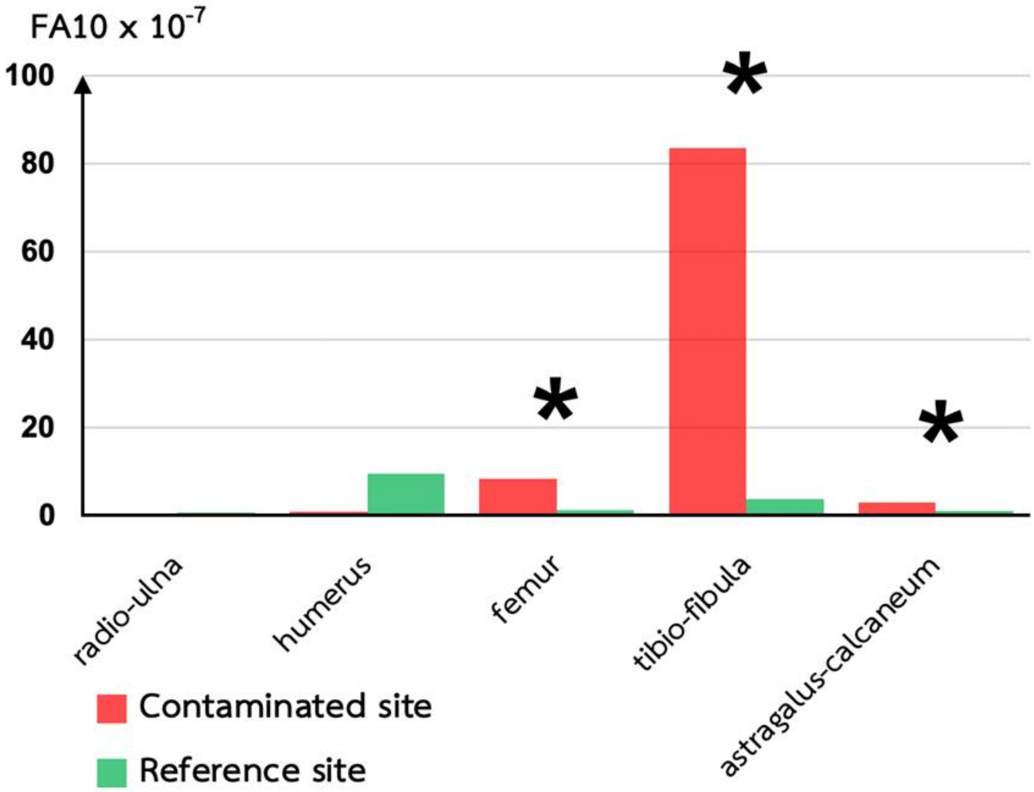

Figure 5.6 Fluctuating asymmetry of bone weight of adult male frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021. Asterisk (*) indicates significant difference between sites, $p \leq 0.05$

2.3. Adult female bone length

2.3.1. Two-way ANOVA to test for FA assumption

Statistical analysis showed that side variation on length of 5 appendage bones was not significant in both sites, suggesting that directional asymmetry (DA) was not present. The individual variation was significant in both sites, meaning that size/shape variation was present. Lastly, the interaction between side and individual variations suggests a presence of FA (Table 5.5). Thereby, the FA on length of 5 appendage bones of adult female frogs can be determined.

Table 5.5 Two-way ANOVA on bone length of adult female frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Female bone length	Contaminated site		Reference site	
(*n* Contaminated: Reference)	Mean square	*p*-value	Mean square	*p*-value
Radio-ulna (44: 57)				
Side variation	1.20×10^{-3}	> 0.05	1.04×10^{-2}	> 0.05
Individual variation	3.92	≤ 0.05	3.67	≤ 0.05
Interaction (sid. x ind.)	2.60×10^{-2}	≤ 0.05	3.22×10^{-2}	≤ 0.05
residual	3.00×10^{-4}	–	1.00×10^{-4}	–
Humerus (43: 61)				
Side variation	1.42×10^{-2}	> 0.05	1.90×10^{-2}	> 0.05
Individual variation	9.14	≤ 0.05	9.86	≤ 0.05
Interaction (sid. x ind.)	4.83×10^{-2}	≤ 0.05	5.20×10^{-2}	≤ 0.05
residual	4.00×10^{-4}	–	1.00×10^{-4}	–
Femur (42: 61)				
Side variation	3.77×10^{-2}	> 0.05	4.74×10^{-2}	> 0.05
Individual variation	22.55	≤ 0.05	23.71	≤ 0.05
Interaction (sid. x ind.)	3.62×10^{-2}	≤ 0.05	1.87×10^{-2}	≤ 0.05
residual	1.00×10^{-4}	–	0	–
Tibio-fibula (44: 58)				
Side variation	1.98×10^{-1}	> 0.05	0.21×10^{-1}	> 0.05
Individual variation	35.04	≤ 0.05	36.47	≤ 0.05
Interaction (sid. x ind.)	6.30×10^{-2}	≤ 0.05	1.90×10^{-2}	≤ 0.05
residual	0	–	0	–
Astragalus-calcaneum (43: 60)				
Side variation	15.83×10^{-2}	> 0.05	0.21×10^{-2}	> 0.05
Individual variation	9.97	≤ 0.05	10.18	≤ 0.05
Interaction (sid. x ind.)	4.40×10^{-2}	≤ 0.05	1.64×10^{-2}	≤ 0.05
residual	2.00×10^{-4}	–	0	–

2.3.2. Comparison of FA in female bone length between sites

Statistical analysis by comparing FA10 between sites using Fisher-Snedecor distribution revealed that there were significant site-related differences of FA in femur tibio-fibula, and astragalus-calcaneum lengths of adult female frogs, showing higher FA from contaminated site. However, there was no significant site-related difference of FA in radio-ulna, and humerus on adult female frogs (Table 5.6, Figure 5.7).

Table 5.6 Fluctuating asymmetry of bone length of adult female frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Female bone length	**Contaminated site**		**Reference site**		**Statistical**
(*n* Contaminated: Reference)	df	FA	df	FA	**value**
Radio-ulna (*n* 44: 57)	42.11	1.28×10^{-2}	55.67	1.60×10^{-2}	$p > 0.05$
Humerus (*n* 43: 61)	41.30	2.39×10^{-2}	59.84	2.59×10^{-2}	$p > 0.05$
Femur (*n* 42: 61)	40.67	1.80×10^{-2}	59.70	0.93×10^{-2}	$p \leq 0.05$
Tibio-fibula (*n* 44: 58)	42.71	3.13×10^{-2}	56.71	0.93×10^{-2}	$p \leq 0.05$
Astragalus-calcaneum (*n* 43: 60)	41.59	2.18×10^{-2}	58.64	0.81×10^{-2}	$p \leq 0.05$

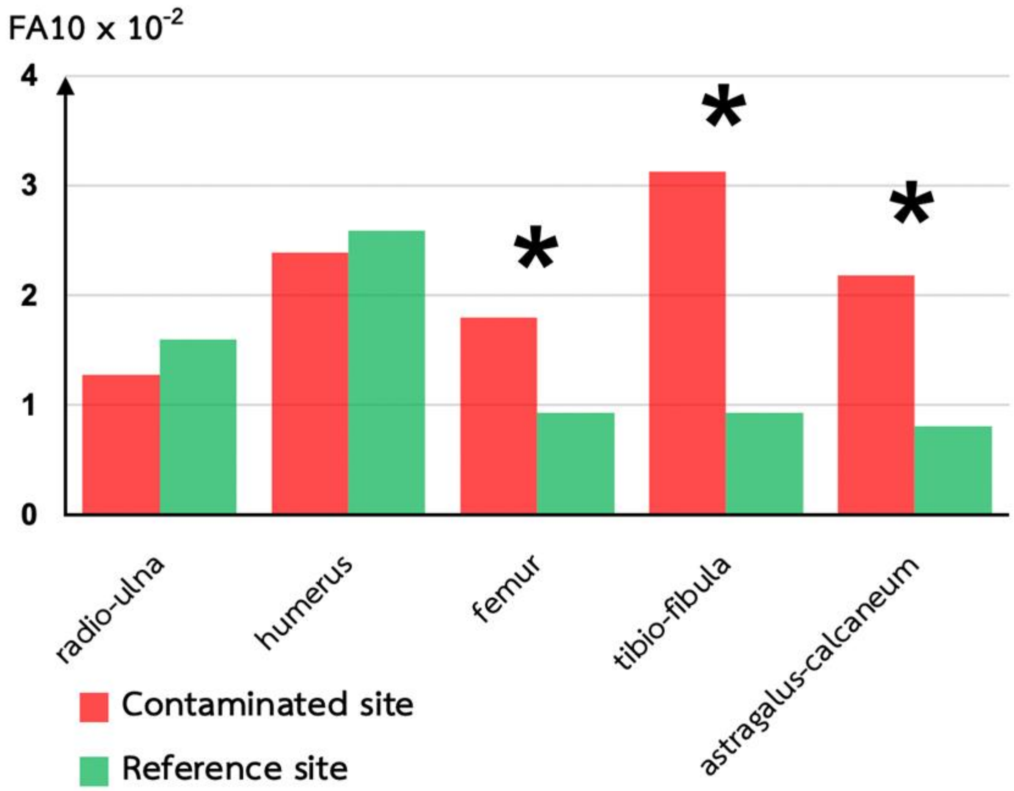

Figure 5.7 Fluctuating asymmetry of bone length of adult female frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021. Asterisk (*) indicates significant difference between sites, $p \leq 0.05$

2.4. Adult female bone weight

2.4.1. Two-way ANOVA to test for FA assumption

Statistical analysis showed that side variation on weight of 4 appendage bones was not significant in both sites, suggesting that directional asymmetry (DA) was not present. Side variation on weight of humerus bone was significant in contaminated site, however, comparison of DA and FA4a suggesting that DA was not interfering with FA. The individual variation was significant in both sites, meaning that size/shape variation was present. Lastly, the interaction between side and individual variations suggests a presence of FA (Table 5.7). Thereby, the FA on weight of 5 appendage bones of adult female frogs can be determined.

Table 5.7 Two-way ANOVA on bone weight of adult female frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Female bone weight	Contaminated site		Reference site	
(*n* Contaminated: Reference)	Mean square	*p*-value	Mean square	*p*-value
Radio-ulna (43: 57)				
Side variation	1.28×10^{-7}	> 0.05	4.47×10^{-7}	> 0.05
Individual variation	3.44×10^{-5}	≤ 0.05	3.04×10^{-5}	≤ 0.05
Interaction (sid. x ind.)	2.23×10^{-7}	≤ 0.05	1.40×10^{-7}	≤ 0.05
residual	1.20×10^{-8}	–	1.59×10^{-8}	–
Humerus (42: 61)				
Side variation	3.17×10^{-6}	≤ 0.05	2.02×10^{-6}	> 0.05
Individual variation	1.40×10^{-4}	≤ 0.05	1.59×10^{-4}	≤ 0.05
Interaction (sid. x ind.)	6.29×10^{-7}	≤ 0.05	6.58×10^{-7}	≤ 0.05
residual	1.00×10^{-8}	–	1.50×10^{-8}	–
Femur (42: 55)				
Side variation	4.37×10^{-6}	> 0.05	2.82×10^{-6}	> 0.05
Individual variation	5.93×10^{-4}	≤ 0.05	6.45×10^{-4}	≤ 0.05
Interaction (sid. x ind.)	2.17×10^{-6}	≤ 0.05	1.97×10^{-6}	≤ 0.05
residual	1.00×10^{-8}	–	1.00×10^{-8}	–
Tibio-fibula (44: 55)				
Side variation	1.40×10^{-5}	> 0.05	0.51×10^{-5}	> 0.05
Individual variation	1.05×10^{-3}	≤ 0.05	1.08×10^{-3}	≤ 0.05
Interaction (sid. x ind.)	1.34×10^{-5}	≤ 0.05	0.37×10^{-5}	≤ 0.05
residual	1.00×10^{-8}	–	1.00×10^{-8}	–
Astragalus-calcaneum (42: 58)				
Side variation	4.82×10^{-7}	> 0.05	0.18×10^{-7}	> 0.05
Individual variation	1.75×10^{-4}	≤ 0.05	1.84×10^{-4}	≤ 0.05
Interaction (sid. x ind.)	8.90×10^{-7}	≤ 0.05	6.01×10^{-7}	≤ 0.05
residual	1.20×10^{-8}	–	1.40×10^{-8}	–

2.4.2. Comparison of FA in female bone weight between sites

Statistical analysis by comparing FA10 between sites using Fisher-Snedecor distribution revealed that there were significant site-related differences of FA in radio-ulna and tibio-fibula weights of adult female frogs, showing higher FA from contaminated site. However, there was no significant site-related difference of FA in humerus, femur, and astragalus-calcaneum of adult female frogs (Table 5.8, Figure 5.8).

Table 5.8 Fluctuating asymmetry of bone weight of adult female frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Female bone weight	**Contaminated site**		**Reference site**		**Statistical**
(*n* Contaminated: Reference)	df	FA	df	FA	**value**
Radio-ulna (*n* 43: 57)	37.66	1.05×10^{-7}	43.28	0.62×10^{-7}	$p \leq 0.05$
Humerus (*n* 42: 61)	39.66	3.04×10^{-7}	57.20	3.21×10^{-7}	$p > 0.05$
Femur (*n* 42: 55)	40.59	10.78×10^{-7}	53.18	9.78×10^{-7}	$p > 0.05$
Tibio-fibula (*n* 44: 55)	42.92	66.92×10^{-7}	53.64	18.40×10^{-7}	$p \leq 0.05$
Astragalus-calcaneum (*n* 42: 58)	39.93	4.39×10^{-7}	54.37	2.93×10^{-7}	$p > 0.05$

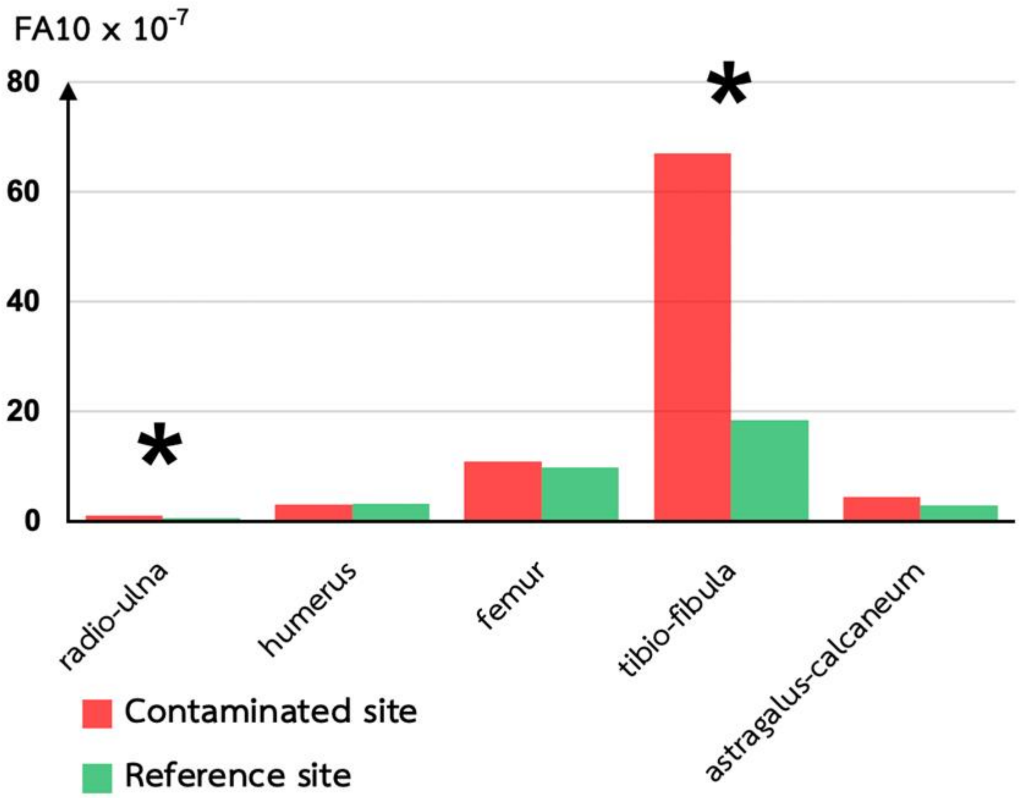

Figure 5.8 Fluctuating asymmetry of bone weight of adult female frogs *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021. Asterisk (*) indicates significant difference between sites, $p \leq 0.05$

3. Size-frequency distribution

Overall data of adult males and adult females showed a higher means of SVL from contaminated site (38.30 ± 1.186 mm) than those in reference site (37.87 ± 1.809 mm). Based on the male frogs, the statistical analysis showed that there was significant site-related difference (two-sample Kolmogorov-Smirnov test, D = 0.024, $p \leq 0.05$). Based on the female frogs, the statistical analysis showed that there was significant site-related difference (two-sample Kolmogorov-Smirnov test, D = 0.613, $p \leq 0.05$). Based on the whole population, the statistical analysis showed that there was significant site-related difference (two-sample Kolmogorov-Smirnov test, D = 0.029, $p \leq 0.05$). Disproportionate distribution was observed in contaminated sites (Figure 5.9).

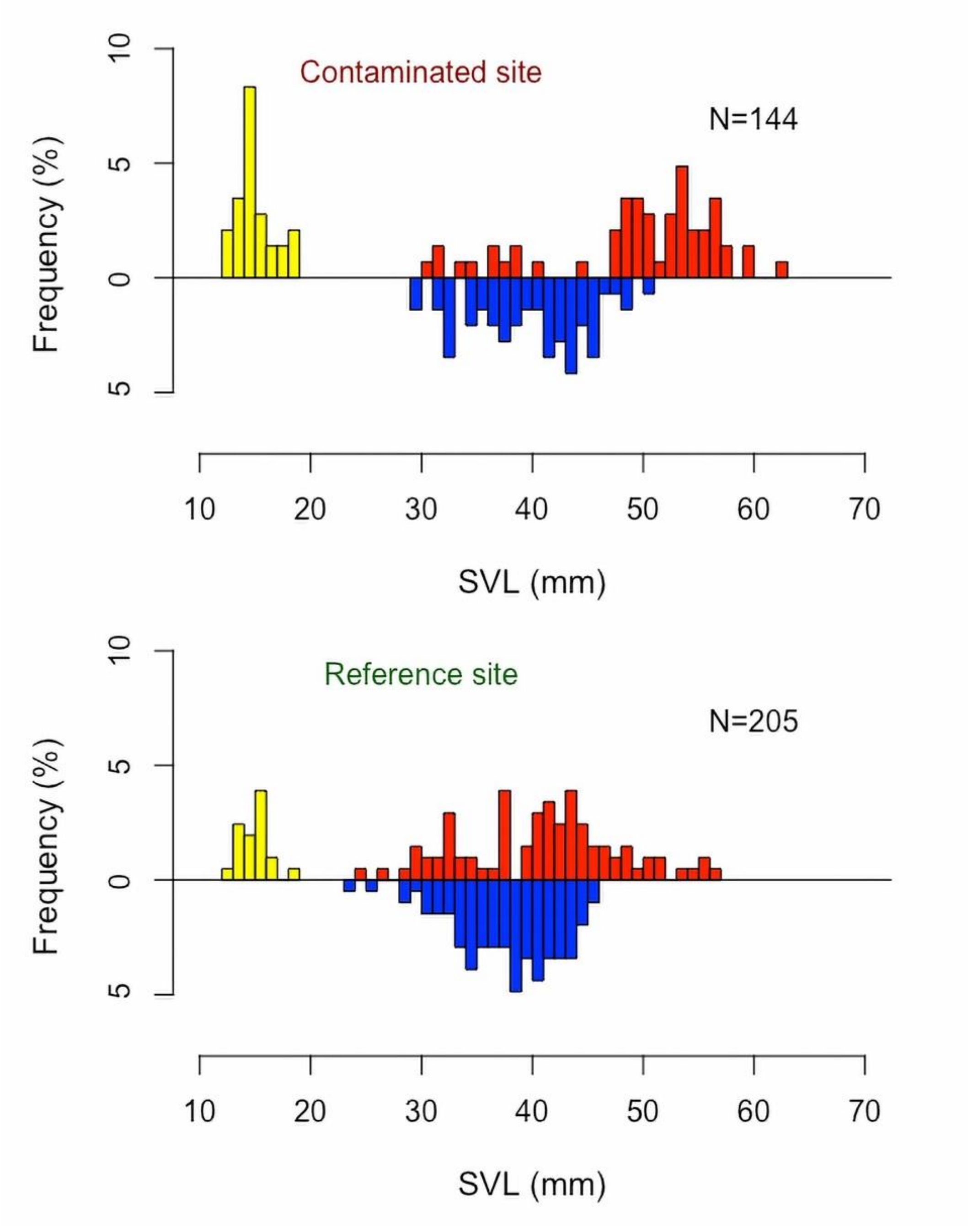

Figure 5.9 Size-frequency distribution (in percentage) of rice frog *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021. Yellow bar, red bar, and blue bar correspond to froglets, female frogs, and male frogs, respectively.

Discussions

To date, there are limited studies that focus on the adverse effect of herbicide on amphibians based on population parameters. The result on growth pattern was in line with the previous report, showing the disruption of the growth and development in a variety of amphibian species: relatively low concentration of atrazine, glyphosate, 2,4-D, and triadimefon results in adverse effect on African claw frog organo-morphogenesis (Lenkowski et al., 2010); herbicides contaminant influences the growth pattern of rice frogs living in the paddy field by showing difference growth slopes (Thammachoti et al., 2012). In light of these reports, it can be concluded that the difference in growth patterns is likely influenced by herbicide contamination either directly through herbicide exposure or indirectly through other means such as food availability in the areas (Hirai and Matsui, 2001).

Results in fluctuating asymmetry showed that FA was present in every appendage bone of forelimb (radio-ulna and humerus) and hindlimb (femur, tibio-fibula, astragalus-calcaneum) of the rice frogs living in both reference and contaminated sites. The presence of FA on both sites may be due to similar geographic and climatic conditions. Comparison of FA between sites using bone length and bone weight suggest that FA from contaminated site were significantly higher than those in reference site. It was assumed that various environmental stressor may negatively affect developmental homeostasis, thus, showing higher fluctuating asymmetry (Palmer and Strobeck, 1986). In comparison to length parameters, it seems that weight parameters provide stronger evidence by reflecting a significant increase of FA on various appendage bones. The possibility is that the bones naturally lighten with age yet keep their length, which might be induced by environmental stressors such as agrochemical exposure (Söderman et al., 2007).

The results of FA analysis were similar to the previous report from various amphibian species that associated with various environmental stressors, e.g., logging activity (Lauck, 2006), acid environment (Söderman et al., 2007), habitat loss (Eterovick et al., 2016), anthropogenic pollution (Zhelev et al., 2015a), polluted river (Zhelev et al., 2019), farming and intensive farmland (Guillot et al., 2016), and intensive herbicide utilization (Thammachoti, 2012). It is noteworthy that adverse effect on the development and growth of amphibians has been reportedly caused by paraquat (Osano et al., 2002) and glyphosate (Bach et al., 2016; Babalola et al., 2019). The utilization of these herbicide mixtures (i.e., atrazine, glyphosate, and paraquat) may be responsible for increased environmental stressors, leading to higher developmental instabilities in frogs from contaminated site. Moreover, the result on FA may also correspond to the presence of herbicides contamination, as previously observed on herbicide residue analysis (Chapter III). It can be suggested that fluctuating asymmetry may provide a useful tool to monitor the impact of environmental stressors on amphibian populations.

Previous investigation of FA on forelimb (radio-ulna and humerus) and hindlimb (femur and tibio-fibula) of rice frog *F. limnocharis* (Thammachoti, 2012) showed that there was a significantly higher FA of humerus, femur, and tibio-fibula from contaminated site which are similar to the results of this study. The lesser amount of FA in forelimb might be due to differences in appendage bones development. The development of appendages in frogs is started during tadpole phase where distal bones (tibio-fibula and radio-ulna) develop later than proximal bones (humerus and femur) (Gilbert and Barresi, 2016). Since the budding of hindlimb was started earlier than forelimb (Duellman and Trueb, 1994), it may result in a longer exposure time to an environmental stressor, thus, showing a higher FA in hindlimb compared to forelimb. In addition to the previous results (Thammachoti, 2012), this study examined a higher FA in astragalus-calcaneum from contaminated site which may provide an alternative trait to monitor the impact of herbicides exposure in amphibians' development.

It was previously hypothesized that the pattern of fluctuating asymmetry differed between forelimb and hindlimbs in amphibians, possibly due to the unequal roles in movement and locomotor style (Didde and Rivera, 2019). The reason is that the hindlimb (mostly for jumping) would express lower FA than forelimb (only for landing) in amphibians, due to the natural selection favoring the most important trait (Didde and Rivera, 2019). However, this study does not support this notion since higher FA was observed mostly on the hindlimb (femur, tibio-fibula, and astragalus-calcaneum) of rice frogs. In addition, it is known that forelimb is also an important morphological trait for mating in frogs (Söderman et al., 2007). Consequently, the higher FA in forelimb (radio-ulna) of adult female frogs may reduce the reproductive fitness of the population in the future. The higher FA on hindlimb (femur, tibio-fibula, and astragalus-calcaneum) in rice frogs from contaminated sites may indicate a decrease in moving and escaping capabilities. Since frogs utilized saltatorial locomotion for foraging and survival (Duellman and Trueb, 1994), any disruption on hindlimb will greatly impair their fitness in the population. Significant differences in FA of femur between sites on both adult male and adult female frogs may also suggest a role of endocrine-disrupting chemicals (e.g., atrazine) in exerting its estrogenic effect on bone development (Agas et al., 2013). It was stated that estrogen receptors were found in cells that are responsible for limb bone ossification (Gilbert and Barresi, 2016). It can be suggested the higher FA from contaminated site may be affected by intensive herbicide utilization.

Prior studies using skeletochronology (Kusrini, 2005; Othman, 2009; Thammachoti, 2012) have failed to demonstrate the presence of the line of arrested growth (i.e., age estimation) in the bone of rice frogs. Therefore, size-frequency distribution may provide an alternative option to investigating the adverse effect of herbicides on amphibians' populations.

Results on size-frequency distributions showed that there was a negative impact of herbicide on the population structure of rice frogs, by showing significant site-related differences. The gaps in the frequency distribution, particularly for frogs with SVL of 20–30 mm (froglet–juvenile, Figure 5.9) might be explained by the rice frogs' survival strategies, which included explosive breeding that was strongly linked with certain rainfall patterns (Duellman and Trueb, 1994). As a consequence, a cohort of frogs on particular life stages was not encountered during the sampling. Previous studies on the population structure of *F. limnocharis-iskandari* complex in Indonesia (Kusrini, 2005), showed that the population fluctuated along with the paddy cultivating cycle, which corresponded to the timing of recruitment which occurs simultaneously with water. However, it was stated that continuous pesticide exposure did not appear to influence the rice frog population structure (Kusrini, 2005). In contrast, the results from the current study showed a significant site-related difference in population structures and disproportionate distribution, possibly due to the different degrees of herbicides exposure. Further investigation showed that agrochemicals may induce immunotoxicity effect resulting in higher parasite load (Köhler and Triebskorn, 2013), where newly metamorphosing frogs are more sensitive (Rollins-Smith et al., 2006); and endocrine-disrupting chemicals along the suburbanization area may result in reproductive failure and ultimately shifts sex ratios in the population (Lambert et al., 2015). It can be suggested that the difference in size-frequency distribution may be affected by the combination of herbicide mixture.

Conclusion

In the last segment of this study, there was a significant site-related difference in growth patterns, indicating the detrimental impact of herbicide contamination on rice frog's growth. Significant site-related difference of fluctuating asymmetry was found on (primarily) hindlimb of rice frog's appendage bones, where the measurement on weight gives stronger evidence rather than length, suggesting the negative impact on frog's developmental stability. There was significant site-related difference in size-frequency distribution and disproportionate distribution in contaminated sites, indicating to the influence of herbicides on population structures. It can be suggested that extensive and continuous herbicide utilization may influence the non-target organism at the population scale.

CHAPTER VI

GENERAL CONCLUSION AND RECOMMENDATION

In this present study, the potential impact of herbicide utilization was investigated on the rice frogs living in the paddy field. Nan Province in the northern part of Thailand was chosen as a study site since there are many agricultural areas with intensive herbicide utilization, i.e., atrazine, glyphosate, paraquat. The rice frog *Fejervarya limnocharis* was used as sentinel species due to their susceptibility and sensitivity where multiple parameters on tissues, organismal, and population parameters were examined.

The potentially contaminated site is a paddy field with intensive herbicide utilization, while the reference site is a paddy field with no history of herbicide utilization and is separated as far as 7 km apart by a major river. Apart from the difference in history of herbicide utilization, these sites have similar geographic and climatic conditions, and their effects on frogs' populations should be minimum.

Samplings were conducted during July and October 2020, and February 2021 covering the seasonal period (wet-dry season) and agricultural cultivation cycle. Subsequently, it was subjected to analysis using multiple parameters. Biomonitoring employs three categories of biomarkers: 1) biomarkers of exposure—measurement of exposure in environment and body, 2) biomarkers of effect—measurement of any clinical effects, and 3) biomarkers of susceptibility—indicate the individuals with less survival chance; were carried out. The results were divided into three main parts, i.e., herbicide contamination, organismal parameter, and population parameter (Table 6.1).

In the first part of this study, herbicide residues (i.e., atrazine, glyphosate, paraquat) were analyzed in composited water samples and frog tissues which were collected during February 2021 and compared between sites. It showed that detectable amounts of atrazine (1.39 ng/mL) were found in contaminated site, but not in reference site, whereas the level of glyphosate and paraquat in water were below the limit of detection. Three herbicide residues (i.e., atrazine, glyphosate, paraquat) were found on the tissue of rice frogs at both sites, with a higher concentration mean of glyphosate and paraquat from contaminated site. Interestingly, paraquat residues were found far beyond the maximum residue limit allowed in food, meaning that the farmers in the areas continue to use banned herbicides (i.e., paraquat), and the results raise concern over the consumption of the frogs. There was evidence of varying degrees of herbicide contamination in the environment and on body, which corresponded to the biomarkers of exposure categories.

In the second part of this study, the adverse effects of herbicides utilization were investigated based on organismal parameters, i.e., gonad weight, liver weight, and body weight, and compared between sites. It revealed that there was a higher ovarian weight of rice frogs from contaminated site, indicating a potential negative effect of xenoestrogen. In addition, there was a site-related difference in liver weight of rice frogs, possibly due to exposure to xenobiotics. Furthermore, there was a site-related difference in body weight of rice frogs, which may be contributed by liver and ovarian weights. It can be concluded that continuous and intensive utilization of herbicides in paddy fields may induce morphological alterations in rice frogs, which corresponded to the biomarkers of effect and susceptibility categories.

In the last part of this study, the adverse effect of herbicide contamination—as a significant environmental stressor, was examined based on population parameters, i.e., growth pattern, fluctuating asymmetry, and size-frequency distribution, and compared between sites. There was a significant difference in growth patterns between sites, indicating the influence of herbicide contamination on rice frogs' growth. Fluctuating asymmetry—as a proxy of developmental instability, showed that utilization of herbicides increases the fluctuating asymmetry in (primarily) hindlimb of frog's appendage bones from contaminated site, indicating a detrimental impact on frog's developmental stability. Significant difference was observed on size-frequency distribution between sites where disproportionate distribution was evidenced in contaminated site, indicating the influence of herbicide on rice frog's population structure. It can be suggested that intensive and continuous herbicide utilization may influence the non-target organism at the population levels, which corresponded to the biomarkers of effect and susceptibility categories.

In general, biomonitoring using biomarkers of exposure, effect, and susceptibility based on herbicide contamination, organismal, and population factors revealed that herbicide utilization may have a negative effect on health of rice frog *F. limnocharis,* which may lead to subtle and perpetual changes to overall paddy field ecosystems. The findings of this study could serve as a forewarning of potential environmental health hazards for vertebrates living in close proximity to herbicide use areas, including humans.

Recommendations

Throughout this study, several modifications were made to aid in the improvement of data collecting and analysis. The section that follows offers many evaluations of this finding. Additionally, the list contains opportunity for future research based on the present study's results.

1. **Frog's collection:** There are differences in the covered sampling areas, which must be estimated in future population studies. While the current study was based on three observation periods, monthly interval observation will surely yield a reliable pattern representing herbicide exposure across several parameters. Additional approaches, such as the mark-recapture method, is recommended to determine the population size at the study site.
2. **Contaminant analysis:** Other agrochemical pollutants (e.g., pesticides, fungicides, and fertilizers) may also present in the study site and need to be investigated in future studies. One novel approach is to correlate herbicide residues with individual frogs' body weight or snout-vent length, revealing that herbicides may behave differently at different body sizes.
3. **Morphological parameters:** Due to the ontogeny of the rice frog, another approach, such as ANCOVA analysis, may be utilized to address these problems. To minimize bias in the morphological results, it is recommended that only adult rice frogs be utilized to accurately reflect the detrimental impact of pesticide contamination.
4. **Population parameters:** Size-frequency distributions may be used to investigate detrimental impacts of herbicides on population structures. However, there are

limited studies have been conducted on the negative impact of herbicides on populations. Due to the rice frog's ontogeny, adult frogs should be used to assess the herbicide's influence on fluctuating asymmetry. Additional bones, such as phalanges and carpal bones, might be employed as a trait in fluctuating asymmetry analysis in the future.

Future research

Additional herbicide analysis of frogs at different developmental stages may provide an insight on the nature of herbicide residue biomagnification and/ or bioaccumulation. Histopathological evaluation on organs of concern may validate the herbicide's impact on rice frogs and may resolved the discrepancy in the current study's findings. Future research should emphasize on hatchling ratios, mortality and natality rates, sex ratios, and age structure in order to determine the effect of herbicide on the frog's population's survival.

Table 6.1 Summary in multiple parameters between sites to monitor the influence of herbicide on health status of rice frog *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand

Contaminant analysis	Values		Remarks
	Contaminated site	Reference site	
Water			
Atrazine	1.39 ng/mL	N/D	–
Glyphosate	N/D	N/D	–
Paraquat	N/D	N/D	–
Frog tissue			
Atrazine	1.60 ± 0.26	1.27 ± 0.24	No significant difference between sites
Glyphosate	26.05 ± 5.83	6.19 ± 0.61	No significant difference between sites
Paraquat	115.89 ± 47.11	27.98 ± 8.91	Significant difference between sites

Remarks:

(N/D) Not detected or contamination levels lower than the limit of detection

Table 6.1 Summary in multiple parameters between sites to monitor the influence of herbicide on health status of rice frog *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand (continued)

Organismal parameters	Values		Remarks
	Contaminated site	Reference site	
Testicular weight			
July 2020	0.015 ± 0.001	0.014 ± 0.001	No significant difference between sites
October 2020	0.002 ± 0.001	0.005 ± 0.001	No significant difference between sites
February 2021	0.012 ± 0.001	0.011 ± 0.001	No significant difference between sites
Ovarian weight			
July 2020	0.879 ± 0.052	0.681 ± 0.082	No significant difference between sites
October 2020	0.067 ± 0.003	0.072 ± 0.003	No significant difference between sites
February 2021	0.443 ± 0.123	0.547 ± 0.123	No significant difference between sites
Liver weight			
July 2020	0.177 ± 0.005	0.166 ± 0.005	No significant difference between sites
October 2020	0.122 ± 0.013	0.136 ± 0.016	No significant difference between sites
February 2021	0.096 ± 0.005	0.115 ± 0.005	Significant difference between sites
Body weight			
July 2020	6.971 ± 0.182	6.382 ± 0.123	Significant difference between sites
October 2020	7.212 ± 0.227	7.390 ± 0.245	No significant difference between sites
February 2021	5.596 ± 0.217	5.750 ± 0.145	No significant difference between sites

Table 6.1 Summary in multiple parameters between sites to monitor the influence of herbicide on health status of rice frog *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand (continued)

Population parameters	Values		Remarks
	Contaminated site	Reference site	
Growth Pattern (Sc)	2.8371	2.6569	Significant difference between sites
Fluctuating asymmetry			
Male bone length			
Radio-ulna	1.20×10^{-2}	3.49×10^{-2}	No significant difference between sites
Humerus	2.45×10^{-2}	3.43×10^{-2}	No significant difference between sites
Femur	7.24×10^{-2}	1.32×10^{-2}	Significant difference between sites
Tibio-fibula	2.72×10^{-2}	3.01×10^{-2}	No significant difference between sites
Astragalus-calcaneum	1.97×10^{-2}	1.03×10^{-2}	Significant difference between sites
Male bone weight			
Radio-ulna	0.33×10^{-7}	0.62×10^{-7}	No significant difference between sites
Humerus	0.84×10^{-7}	9.39×10^{-7}	No significant difference between sites
Femur	8.31×10^{-7}	1.26×10^{-7}	Significant difference between sites
Tibio-fibula	83.40×10^{-7}	3.75×10^{-7}	Significant difference between sites
Astragalus-calcaneum	2.88×10^{-7}	1.09×10^{-7}	Significant difference between sites

Table 6.1 Summary in multiple parameters between sites to monitor the influence of herbicide on health status of rice frog *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand (continued)

Population parameters	Values		Remarks
	Contaminated site	Reference site	
Female bone length			
Radio-ulna	1.28×10^{-2}	1.60×10^{-2}	No significant difference between sites
Humerus	2.39×10^{-2}	2.59×10^{-2}	No significant difference between sites
Femur	1.80×10^{-2}	0.93×10^{-2}	Significant difference between sites
Tibio-fibula	3.13×10^{-2}	0.93×10^{-2}	Significant difference between sites
Astragalus-calcaneum	2.18×10^{-2}	0.81×10^{-2}	Significant difference between sites
Female bone weight			
Radio-ulna	1.05×10^{-7}	0.62×10^{-7}	Significant difference between sites
Humerus	3.04×10^{-7}	3.21×10^{-7}	No significant difference between sites
Femur	10.78×10^{-7}	9.78×10^{-7}	No significant difference between sites
Tibio-fibula	66.92×10^{-7}	18.40×10^{-7}	Significant difference between sites
Astragalus-calcaneum	4.39×10^{-7}	2.93×10^{-7}	No significant difference between sites
Size frequency distribution (SVL)	38.30 ± 1.186	37.87 ± 1.809	Significant difference between sites

APPENDICES

Appendix A

Rice cultivation period in Nan Province, Thailand

Rice cultivation period in Nan Province, Thailand

In Nan Province, Thailand, the basic rice cultivation time is divided into three distinct seasons: planting season, growth season, and harvest season. Because agricultural approaches (1-crop cycle vs. 2-crop cycle) varies, the agricultural cultivation cycle likewise changes. The agricultural cultivation cycle variations between these two rice production approaches in Nan Province are presented in Table A.1.

Table A.1 Rice cultivation period in Nan Province, Thailand (Sakchai Korkerd, interview, 1 November 2021)

Practice	Agricultural cultivation period											
	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
1-crop cycle	Planting season	Growing season			Harvest season		Fallow season or alternative crop *(corn/ sesame)*					
2-crop cycle	Growing season			Harvest season		Planting season	Growing season			Harvest season		Planting season

Appendix B

General climate description in Nan Province during sampling period

(April 2020–June 2021)

General climate description in Nan Province during sampling period

In this study period (April 2020–June 2021), the average air temperature was 27.45°C and average total rainfall was 112.19 mm. The demarcation of wet and dry seasons in this study was determined based on the climate diagram plot between mean temperature and total rainfall of each month (Walter et al., 1975). The climate during this sampling period was listed below (Figure B.1):

1. Early wet season (April 2020–June 2020)
2. Late wet season (July 2020–September 2020)
3. Early dry season (October 2020–December 2020)
4. Late dry season (January 2021–March 2021)

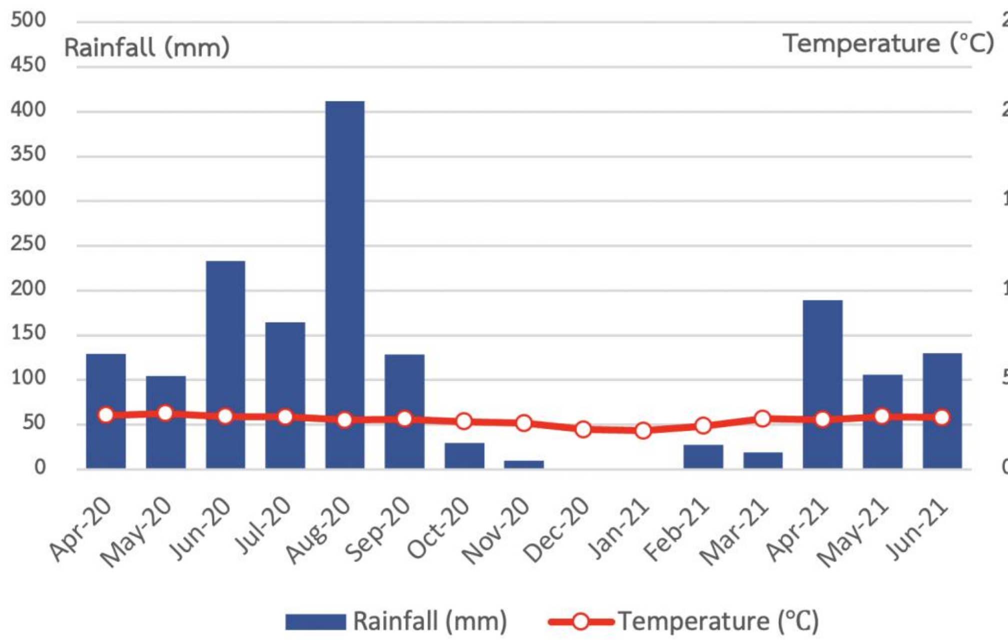

Figure B.1 Climograph of Nan Province during sampling period (April 2020–June 2021)

Appendix C

Herbicide residue statistical results on the sex-related differences in reference site and contaminated site
(February 2021)

Sex-related difference on herbicide residue analysis

Based on February 2021 data, the results showed that there was no significant sex-related difference between male and female frogs both in atrazine, glyphosate, and paraquat residues (Table C.1). Therefore, the male and female data were pooled for the further analysis.

Table C.1 Statistical results on the sex-related differences of herbicide residue in reference site and contaminated site

Herbicides	**Tissue residue in dry weight (ng/g)**		**Power of test** (α= 0.05)
	Male (*n*: 5) (Mean ± SEM)	**Female (*n*: 5)** (Mean ± SEM)	
Atrazine			
Contaminated site[a]	1.58 ± 0.34	1.63 ± 0.43[ns]	0.05
Reference site[a]	1..53 ± 0.44	1.02 ± 0.17[ns]	0.06
Glyphosate			
Contaminated site[b]	21.11 ± 11.57	10.98 ± 2.52[ns]	0.05
Reference site[a]	6.97 ± 1.07	5.41 ± 0.52[ns]	0.11
Paraquat			
Contaminated site[b]	120.21 ± 71.79	111.58 ± 69.46[ns]	0.05
Reference site[b]	39..35 ± 15.95	16.62 ± 6.21[ns]	0.11

Remarks:

[a] Compared by Student's *t*-test

[b] Compared by Mann-Whitney rank-sum test

[ns] No significant difference between sexes; $p > 0.05$

Appendix D

Collected sample size of rice frogs *Fejervarya limnocharis* during the study (July 2020–February 2021)

Collected sample size of rice frog *Fejervarya limnocharis* during the study

A total of 380 frogs were collected during July 2020–February 2021 sampling (Table D.1). There was a fluctuation of frog abundance throughout sampling period, where July 2020 (late wet season) was the period with the highest encountered frogs (n = 203).

Table D.1 Sample size of rice frog *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, collected during July 2020–February 2021

Month/Year	Period	Contaminated site	Reference site
July 2020	Late wet season,	Froglet = 26	Froglet = 21
	Crop growing period	Juvenile = –	Juvenile = –
		Subadult = –	Subadult = 16
		Adult = 54	Adult = 86
October 2020	Early dry season,	Froglet = –	Froglet = –
	Harvest period	Juvenile = 13	Juvenile = 23
		Subadult = 6	Subadult = 4
		Adult = 8	Adult = 8
February 2021	Late dry season,	Froglet = 5	Froglet = –
	Fallow period	Juvenile = 2	Juvenile = 13
		Subadult = 4	Subadult = 7
		Adult = 26	Adult = 58
	Total	**144**	**236** *

Remark:

* Additional samples were collected beyond primary sampling.

Appendix E

Organismal parameter statistical results on the sex-related differences in reference site and contaminated site

(July 2020–February 2021)

Sex-related difference on organismal parameters

1. Liver weight

After controlling the influence of body weight, the statistical analysis showed that there was no significant sex-related difference in liver weight in contaminated site and reference site throughout sampling periods (Table E.1). Therefore, the male and female data were pooled.

Table E.1 ANCOVA analysis on the sex-related differences of liver weight in reference site and contaminated site in Nan Province, Thailand, throughout sampling periods

Period	Liver weight (g) Male (Mean ± SEM)	Liver weight (g) Female (Mean ± SEM)	Statistical value
July 2020			
Contaminated site[a]	0.242 ± 0.012 (*n*: 20)	0.264 ± 0.009[ns] (*n*: 28)	$F_{1,45}$ = 1.465, p = 0.233, Power of test (α = 0.05) = 0.220
Reference site[a]	0.091 ± 0.003 (*n*: 35)	0.091 ± 0.006[ns] (*n*: 14)	$F_{1,46}$ = 0.022, p = 0.882, Power of test (α = 0.05) = 0.052
October 2020			
Contaminated site[a]	0.080 ± 0.007 (*n*: 6)	0.117 ± 0.017[ns] (*n*: 2)	$F_{1,5}$ = 2.638, p = 0.165, Power of test (α = 0.05) = 0.263
Reference site[a]	0.198 ± 0.016 (*n*: 4)	0.142 ± 0.026[ns] (*n*: 2)	$F_{1,3}$ = 2.597, p = 0.205, Power of test (α = 0.05) = 0.208
February 2021			
Contaminated site[a]	0.089 ± 0.007 (*n*: 10)	0.104 ± 0.007[ns] (*n*: 10)	$F_{1,17}$ = 2.150, p = 0.161, Power of test (α = 0.05) = 0.283
Reference site[a]	0.117 ± 0.006 (*n*: 11)	0.111 ± 0.007[ns] (*n*: 10)	$F_{1,18}$ = 0.444, p = 0.514, Power of test (α = 0.05) = 0.097

Remarks:

[a] Compared by ANCOVA and expressed as estimated marginal means adjusted for body weight

[ns] No significant difference between sexes; p > 0.05

2. Body weight

After controlling the influence of snout-vent length, the statistical analysis showed that there was no significant sex-related difference in body weight in contaminated site and reference site throughout sampling periods, except for reference site during July 2020 and contaminated site during October 2020 (Table E.2). Therefore, the male and female data were pooled.

Table E.2 ANCOVA analysis on the sex-related differences of body weight in reference site and contaminated site in Nan Province, Thailand, throughout sampling periods

Period	Body weight (g)		Statistical value
	Male (Mean ± SEM)	Female (Mean ± SEM)	
July 2020			
Contaminated site[a]	9.275 ± 0.012 (*n*: 24)	9.620 ± 0.230[ns] (*n*: 30)	$F_{1,51}$ = 0.667, p = 0.418, Power of test (α = 0.05) = 0.126
Reference site[a]	4.699 ± 0.131 (*n*: 61)	5.600 ± 0.163[*] (*n*: 41)	$F_{1,99}$ = 17.122, p < 0.001, Power of test (α = 0.05) = 0.984
October 2020			
Contaminated site[a]	9.034 ± 0.584 (*n*: 6)	6.657 ± 0.454[*] (*n*: 8)	$F_{1,11}$ = 5.995, p = 0.032, Power of test (α = 0.05) = 0.607
Reference site[a]	7.030 ± 0.364 (*n*: 4)	6.759 ± 0.252[ns] (*n*: 8)	$F_{1,9}$ = 0.354, p = 0.566, Power of test (α = 0.05) = 0.083
February 2021			
Contaminated site[a]	6.091 ± 0.007 (*n*: 16)	6.932 ± 0.316[ns] (*n*: 13)	$F_{1,26}$ = 3.661, p = 0.067, Power of test (α = 0.05) = 0.453
Reference site[a]	5.359 ± 0.206 (*n*: 36)	5.350 ± 0.239[ns] (*n*: 28)	$F_{1,61}$ = 0.001, p = 0.978, Power of test (α = 0.05) = 0.050

Remarks:

[a] Compared by ANCOVA and expressed as estimated marginal means adjusted for snout-vent length

[*] Significant difference between sexes; $p \leq 0.05$

[ns] No significant difference between sexes; $p > 0.05$

Appendix F

Research Dissemination

1. The 11th Congress of Toxicology in Developing Countries (CTDC11)

Septiadi, L., Thammachoti, P., & Kitana, N. (2021). Herbicides and population health of frog, *Fejervarya limnocharis*, in paddy fields at Northern Thailand. Abstract, the 11th Congress of Toxicology in Developing Countries (CTDC11), June 13-16, 2021, Kuala Lumpur, Malaysia. [Oral Presenter]

Figure F.1 Certificate of participation in CTDC11

<u>Oral Presenter</u>

Figure F.2 Oral presenter poster in CTDC11 international conference

Figure F.3 Presentation slide in CTDC11 international conference

CTDC

11th CONGRESS OF TOXICOLOGY IN DEVELOPING COUNTRIES

Multidisciplinary Approaches in Toxicology Towards Supporting Sustainable Development Goals
Kuala Lumpur, Malaysia 13-16 June 2021

Website:
http://www.ctdc11.org

Organizers:
MySOT Malaysian Society of Toxicology
iutox International Union of Toxicology

25th June 2021

Mr. Luhur Septiadi, Chulalongkorn Uni., Thailand
Ms. Katarina Baralić, Uni. of Belgrade, Serbia
Prof. Mohd. Nazil Salleh, PICOM, Malaysia
Dr. Hisyam Abdul Hamid, UitM, Malaysia
Dr. Salfarina Ramli, UiTM, Malaysia

Dear Presenters, Chair and Moderator,

Oral Session 1: Ecotoxicology / Environmental Toxicology, 14th June 2021

Our deepest gratitude for making the CTDC11 a success. Despite the challenges of virtual networking from home due to the COVID19 pandemic, the Congress has been a great success based on the positive comments we received from the presenters and participants, as well as from the statistics of virtual attendance and viewing.

For your information, we had 260 registered participants of whom 70% are from developing countries, 4% from least developed countries, and 26% from developed countries. Popular topics at the Congress were risk assessment, pesticide management, safety of botanical products, and alternatives to animal testing, reflecting important toxicological issues in developing countries in addressing their respective commitments to the UNSDGs.

Once again, we thank you for your contributions to the success of this event and hope to see you in person at future events.

We also encourage you to submit your work at the Food and Chemical Toxicology Journal. The manuscript submission portal is now open at https://www.editorialmanager.com/fct/default.aspx.
At the 'Select Article Type' dropdown menu please choose "VSI:CTDC11_Toxicology and Sustainability".

Thank you.
Terima kasih dan semoga berjumpa kembali

With best regards,

Dr. Salmaan Hussain Inayat-Hussain
Chair of CTDC11

Dr. Chan Kok Meng
Co-Chair of CTDC11

Open

Contact us: secretary@ctdc11.org

Figure F.4 Letter of appreciation for oral session from CTDC11 international conference

Abstract

11th Congress of Toxicology in Developing Countries
Kuala Lumpur, Malaysia from 13th-16th June 2021

HERBICIDES AND POPULATION HEALTH OF FROG, FEJERVARYA LIMNOCHARIS, IN PADDY FIELDS AT NORTHERN THAILAND

LUHUR SEPTIADI[1], PANUPONG THAMMACHOTI[1], NOPPADON KITANA[1*]

[1]DEPARTMENT OF BIOLOGY, FACULTY OF SCIENCE, CHULALONGKORN UNIVERSITY, BANGKOK 10330, THAILAND

noppadon.k@chula.ac.th

ABSTRACT

Introduction:
Herbicides has been intensively used in Thailand's agriculture, leading to a potential environmental risk to human and non-target organisms (Laohaudomchok et al., 2020). Prior studies using sentinel species (Roy, 2002), showed that frogs living in field with herbicide use in northern Thailand had higher residues and changes in morphological and physiological status (Thammachoti et al., 2012; Jantawongsri et al., 2015).

Objective:
We aimed to examine the potential influence of herbicides on the rice frog Fejervarya limnocharis populations based on morphometric/gravimetric parameters and size-frequency distribution.

Methods:
Samplings of waters and frogs (IACUC of CU-ACUP Review No.2123002) were conducted from two paddy fields with different degree of herbicide utilization at Nan province, northern Thailand, during wet season (July) to dry season (February). Herbicide residues were screened by ELISA. Frogs from these sites were compared for condition factor, hepatosomatic index (HSI), gonadosomatic index (GSI) by two-way ANOVA and Tukey's HSD test; and size-frequency distribution by two-sample Kolmogorov-Smirnov test.

Results and Discussion:
Atrazine was detected in the waters from the contaminated site (Maneein et al., 2011), while atrazine and paraquat tissue residues were markedly different between sites (Jantawongsri et al., 2015). At individual level, condition factor showed significant site-related differences in wet season, indicating potential influence on the overall health. Gravimetric analyses showed significant site-related differences in HSI of both males and females in wet season, indicating a higher exposure to xenobiotics. Although no significant site-related difference was found in GSI of both males and females, it was noteworthy that females from the contaminated site tended to have higher GSI, indicating potential effect of xenoestrogens. At population level, growth patterns were markedly different between sites. Size-frequency distribution showed significant site-related differences and disproportionate distribution of frogs in the contaminated site. Overall results suggest that herbicides could influence non-target organisms at individual and population levels, leading to subtle and perpetual changes towards biodiversity loss in agroecosystem.

Keywords: agrochemicals, gravimetric analysis, morphometric analysis, population, sentinel species

References:

Jantawongsri, K., Thammachoti, P., Kitana, J., et al. (2015). Altered immune response of the rice frog Fejervarya limnocharis living in agricultural area with intensive herbicide utilization at Nan Province. EnvironmentAsia 8(1): 68-74.

Maneein, R., Khonsue, W., Varanusupakul, P., et al. (2011). Association between atrazine utilization and biologic response of rice field crab Esanthelphusa nani in paddy fields of Nan Province, Thailand. Res. J. Chem. Environ. 15(2): 1018-1023.

Roy, D. (2002). Amphibians as environmental sentinels. J. Biosci. 27(3): 187-188.

Thammachoti, P., Khonsue, W., Kitana, J., et al. (2012). Morphometric and gravimetric parameters of the rice frog Fejervarya limnocharis living in areas with different agricultural activity. J. Environ. Prot. Sci. 3(10): 1403-1408.

Figure F.5 Published abstract in CTDC11 international conference

2. The 47th International Congress on Science, Technology, and Technology-based Innovation (STT47) - Sciences for SDGs: Challenges and Solutions

Septiadi, L., Thammachoti, P., Claude, J., & Kitana, N. (2021). Health status of the rice frog *Fejervarya limnocharis* in Nan Province, Thailand, during peak herbicide utilization period. Abstract, the 47th International Congress on Science, Technology, and Technology-based Innovation (STT47) - Sciences for SDGs: Challenges and Solutions, October 5-7, 2021, Kasetsart University, Nakhon Pathom, Thailand. [Oral Presenter]

47th International Congress on Science, Technology and Technology-based Innovation (STT47)
"Sciences for SDGs: Challenges and Solutions"
5-7 October 2021
Wuttichai Kapikran Building, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen Campus
Nakhon Pathom, Thailand

STT47 | KU
KAMPHAENG SAEN

To whom it may concern

This is to certify that Luhur Septiadi
from Chulalongkorn University

attended and presented their work at the 47th Congress on Science, Technology and Technology-based Innovation.

Presentation Title: HEALTH STATUS OF THE RICE FROG Fejervarya limnocharis IN NAN PROVINCE, THAILAND, DURING PEAK HERBICIDE UTILIZATION PERIOD

(Oral Presentation)

The 47th Congress on Science, Technology and Technology-based Innovation (STT47) is jointly organized by the Science Society of Thailand under the Patronage of His Majesty the King, under the theme "Sciences for SDGs: Challenges and Solutions" at Kasetsart University, Kamphangsaen Campus, Nakon Pathom, during 5-7 October, 2021.

Somkiat Ngamprasertsith

Professor Somkiat Ngamprasertsith, Ph.D.
Chairperson STT47

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Secretariat Office, Science Society of Thailand under the Patronage of His Majesty the King
Faculty of Science, Chulalongkorn University, Phya Thai Rd., Bangkok 10330, Thailand.
Tel: 02-252-7987, 0-2218-5245 Fax. 0-2252-4516

Figure F.6 Acceptance letter for participation in STT47 international conference

Oral Presenter

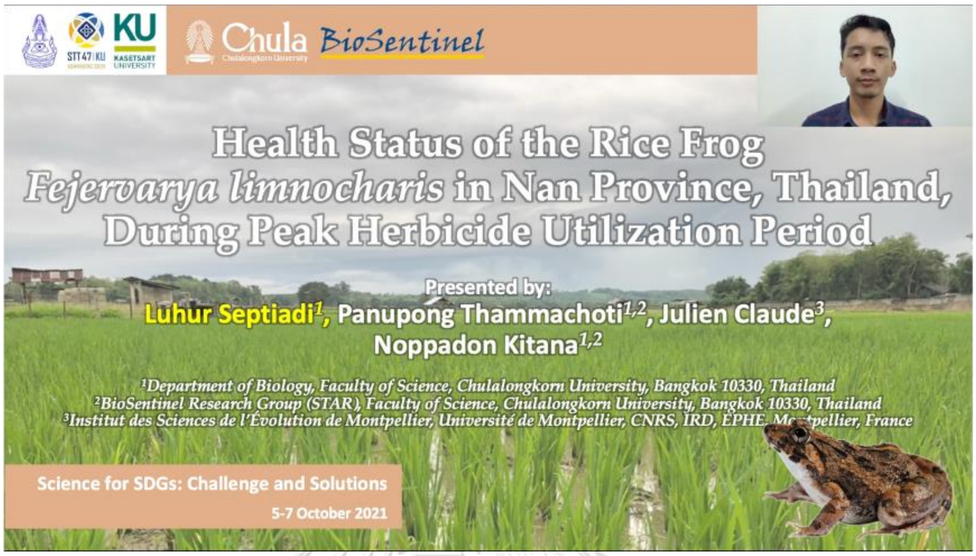

Figure F.7 Presentation slide in STT47 international conference

<u>Proceedings</u>

HEALTH STATUS OF THE RICE FROG *Fejervarya limnocharis* IN NAN PROVINCE, THAILAND, DURING PEAK HERBICIDE UTILIZATION PERIOD

Luhur Septiadi,[1] Panupong Thammachoti,[1,2] Julien Claude,[3] Noppadon Kitana[1,2]*
[1]Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[2]BioSentinel Research Group (STAR), Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[3]Institut des Sciences de l'Évolution de Montpellier, Université de Montpellier, CNRS, IRD, EPHE, Montpellier, France
*e-mail: noppadon.k@chula.ac.th

Abstract:
Herbicides have been continuously and intensively used in Thailand's agriculture, leading to a potential hazard to human and non-target organisms. Prior studies using sentinel species showed that frogs living in herbicide-contaminated paddy fields had higher residues and changes in morphological and physiological status. In this study, we monitor the potential influence of herbicides on health of the rice frog *Fejervarya limnocharis* populations based on organismal parameters (condition factor, gonadosomatic index, hepatosomatic index) and population parameters (growth pattern, size-frequency distribution). Samplings of frogs were conducted from two paddy fields with different degrees of herbicide utilization at Nan province, northern Thailand, during the peak herbicide utilization period (wet season, July 2020). Frogs from these sites were compared and analyzed statistically. The weight-length relationship shows differences in growth patterns between sites. At organismal level, condition factor on adult male frogs showed significant site-related differences. Gonadosomatic index on adult female frogs showed significant site-related differences, indicating potential effect of xenoestrogens. Hepatosomatic index on both sexes shows significant site-related differences, indicating a higher exposure to xenobiotics. At population level, size-frequency distribution showed significant site-related differences and disproportionate distribution. It can be concluded that herbicides could influence non-target organisms at organismal and population levels and may lead to subtle and perpetual changes in agroecosystem health.

Introduction:
Environmental contamination has become one of the serious threats to the global environment. Southeast Asia as the densely populated region utilizing agricultural activity as the backbone of its economy[1], are prone to the health hazards of agrochemicals[2]. Not only affecting many communities[3], but agrochemicals utilization also poses a risk to the non-target organism living in the vicinity due to their persistence which may result in adverse effects[4].

The adverse effects caused by agrochemicals can be monitored on any vertebrates tested in physical conditions and vertebrates living in affected areas. Due to the shared similarities of metabolic process and susceptibility to humans, a variety of vertebrates as sentinel species has been studied before, e.g., mammals[5], turtles[6], fish[7], and amphibians[8]. The observed adverse effect on sentinel species may provide forewarning to the danger of continuous and intensive utilization of agrochemicals.

Amphibians have been considered as one of the most applicable sentinel species due to their susceptibility and sensitivity to environmental change and stressors, especially chemical contaminants during their complex life cycles[9]. For instance, amphibians have been used for study on environmental exposure[10], an animal model for endocrine-disruptor study[11], a model organism for environmental genotoxicity[12], and other physiological studies[13]. Also, a concern was raised on the global decline of amphibians where one of the underlying factors was agrochemicals contaminations, which indirectly affecting amphibian growth and survival in a population[14].

In Thailand, agricultural activities are considered as the foundation of its economy which resulting in the inevitably high demand for imported pesticides[15]. Herbicides make up the largest portion of imported pesticides with the most common type including 2,4 D, ametryn, paraquat, glyphosate, atrazine[15]. Furthermore, it was decided that the use of paraquat is prohibited (along with tightening restrictions on several pesticides) due to its potential effect on health hazards[15]. Nan Province (northern part of Thailand) has become one of the areas with a major agricultural activity which includes crops of paddy, tamarind, and maize. Several efforts to monitor the degree of herbicide impact to a non-target organism in paddy field has been conducted, including in rice field crab[16], freshwater mussel[17], and rice frog[18]. It is important to monitor the adverse effect of herbicide on amphibians living in the affected areas. In this study, we investigate the adverse effect by using rice frog *Fejervarya limnocharis* as sentinel species of agrochemical contamination. We confirmed the impact by using organismal and population parameters, by comparing the frog living in paddy fields with different degrees of herbicide utilization.

Methodology:

Study Sites and Field Sampling

Frogs were collected from contaminated site (a paddy field with intensive herbicide utilization; Loc: 47Q 0686734 UTM 02047312) and reference site (a paddy field with no history of herbicide utilization; Loc: 47Q 0687013 UTM 02054799) in Wiangsa district, Nan Province, Thailand, during 18–19 July 2020. These two sites have similar geographic and climatic conditions but approximately 7 km far away from each other and separated by a big river (Figure 1). It was reported that water from contaminated site has been contaminated by atrazine herbicide[16], and tissue of rice frogs have been exposed to a higher concentration of atrazine, glyphosate, and paraquat herbicides[19]. The highest herbicide utilization was reported in the wet or rainy season (April–July)[18, 20] which corresponds to cultivation period and mean rainfall in Thailand.

Figure 1. Location map showing the contaminated and reference site in Nan Province, Thailand.

Field samplings were conducted using visual encounter survey where several stages of frogs (i.e., froglet, juvenile, sub-adult, adult) were caught by hand at night. To obtain the best comparable data, the survey was restricted to 45 minutes and limited to 4–6 surveyors. The total sample of rice frogs collected from contaminated and reference sites is shown in Table 1.

Table 1. Sample size of rice frog *F. limnocharis* population from contaminated and reference sites in Nan Province, Thailand, during the peak herbicide utilization period

Month Year/ Period	Contaminated Site	Reference Site
July 2020/ Rainy season	Froglet: 26 Juvenile: – Subadult: – Adult: 54	Froglet: 21 Juvenile: – Subadult: 16 Adult: 86
Total	80	123

Estimation of Growth Pattern, Condition Factor, Gonadosomatic index, and Hepatosomatic index

Frogs were immediately transported to the laboratory at Chulalongkorn University Forest and Research Station, Nan Province, and euthanized by immersion in 0.5% tricaine methane sulfonate solution (Sigma-Aldrich, St. Louis, MO, USA). Frogs were measured for body weight (BW) and snout-vent length (SVL) using Ohaus Pioneer Analytical Balances (accuracy 0.0001 g) and Mitutoyo Absolute Digimatic Caliper (accuracy 0.01 mm), respectively. The frogs were dissected and measured for the weight of liver and gonad (testis and ovary) with the aid of Olympus stereomicroscope and Ohaus Pioneer Analytical Balances.

Regression analysis was calculated based on log-transformed data of body weight and snout-vent length represented in Equation (1),

$$\log BW = b \log SVL + \log a \quad (1)$$

where constant b is a scaling coefficient—indicating the growth pattern of population[21], while constant a and b were used for Condition Factor (CF)—the indicator of overall health of frog populations[22], represented in Equation (2).

$CF = (BW \times 100) / (a \times SVL^{b})$ (2)

Gravimetric indices were used to investigate the somatic change on organs, including gonadosomatic index (GSI; relative weight of gonad to body weight) and hepatosomatic index (HSI; relative weight of liver to body weight)[18]. These were estimated based on following Equation (3).

$HSI/GSI = \text{organ weight} \times 100 / \text{body weight}$ (3)

The data based on parameters of organismal level (i.e., CF, GSI, HSI) were represented in boxplot using ggplot2 R-packages[23] analyzed in R v.3.4.1.

Estimation of Size-frequency distribution

The dissected frogs were determined for stage and sex then categorized into froglet, male, and female. The data from each site were represented in relative frequency in percentage using base in R v.3.4.1.

Statistical analysis

Differences in growth patterns were compared between sites by analysis of covariance (ANCOVA) using logSVL as a covariable followed by Bonferroni tests. Data on organism parameters (i.e., CF, GSI, HSI) were priorly tested for normal distribution and homogeneity of variance, and were analyzed separately between sexes. Mean comparisons were analyzed by Student's t-test to investigate the difference between contaminated and reference sites. In case normality and variance from the organismal data were not fulfilled, the data were subjected to Mann-Whitney rank-sum test. The power of test for growth pattern and organismal parameters were also estimated. A two-sample Kolmogorov-Smirnov test was used to test the difference in Size-frequency distribution between contaminated and reference sites.

Results and Discussion:

Weight-length relationship and Growth Pattern

The weight-length relationship (Figure 2) shows that there are differences in growth patterns between sites. The scaling coefficient of frogs from contaminated site (2.8222) was higher than those from reference site (2.5596), indicating different growth pattern between two populations. As the logSVL increases, corresponding logBW also increased (contaminated site: $r = 0.9955$, reference site: $r = 0.9738$). After controlling for the influence of logSVL, ANCOVA showed a significant difference of logBW between sites ($p < 0.05$, power of test $[\alpha = 0.05] = 0.80$). The estimated marginal mean of logBW in frogs from contaminated site (0.495) was significantly higher than those from reference site (0.467), indicating the differences in growth pattern between sites. Differences in growth patterns have been observed from other frog species[24, 25, 26] and within the same frog species[18, 27] exposed to various agrochemical contaminants. It can be concluded that the difference in the growth pattern of the frog population is likely influenced by herbicide contamination.

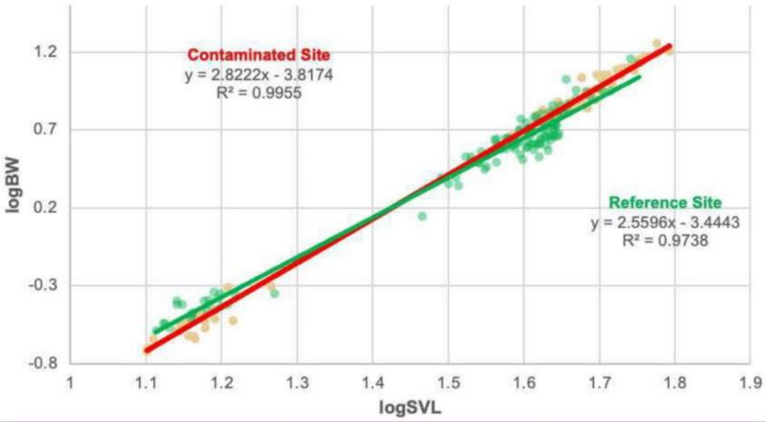

Figure 2. Regression analyses of log-transformed bodyweight (BW) and snout-vent length (SVL) of rice frog *F. limnocharis* population from contaminated and reference site in Nan Province, Thailand, during the peak herbicide utilization period.

Condition Factor, Gonadosomatic Index, and Hepatosomatic Index

Condition factor on adult male frogs from contaminated site (104.69 ± 2.126) was significantly higher than those from reference site (94.78 ± 2.163) (Mann Whitney rank sum test, $p < 0.05$, power of test [α = 0.05] = 0.67). There was no significant difference in CF on adult female frogs between contaminated site (105.18 ± 1.973) and reference site (105.56 ± 2.266) (Student's *t*-test, $p > 0.05$, power of test [α = 0.05] = 0.05). Based on CF results, the differences between contaminated and reference sites were observed only in adult male frogs. Studies from another frog species[22, 28, 29] and within the same frog species[18, 27] generally show that contaminated site has significantly lower CF than those from reference site, indicating frogs from contaminated site have lesser health fitness. The lesser health fitness could imply that the disruption of growth hormone secretion caused by herbicide[24, 30] may lead to smaller frogs, thereby could be easily caught by a predator. In contrast, our study shows high CF observed in adult male frogs living in contaminated sites which does not indicate the herbicide influence on the overall health.

There was no significant different in GSI on adult male frogs between contaminated site (0.30 ± 0.013) and reference site (0.30 ± 0.015) (Student's *t*-test, $p > 0.05$, power of test [α = 0.05] = 0.05), partly due to high individual variation and small sample size. However, GSI on adult female frogs from contaminated site (8.77 ± 0.439) was significantly higher than those from reference site (6.98 ± 1.013) (Mann Whitney rank sum test, $p < 0.05$, power of test ([α = 0.05] = 0.43). Previous studies using the combined dataset of several common frogs shows no site-related difference of GSI in male frogs, except for female frogs from contaminated site which showing significantly higher GSI[31]. It was also similar within the same frog species showing significantly higher GSI of adult female frogs from contaminated site[18]. The larger ovary of frogs from contaminated site is likely influenced by herbicides, as it was previously reported, the higher GSI in adult female frogs of *Fejervarya limnocharis* from Thailand was primarily observed in the wet/rainy season[18, 20] where herbicides (i.e., glyphosate, paraquat, atrazine) were utilized intensively. Atrazine is known as endocrine-disrupting chemicals (EDCs)[32] capable to interrupts the hormonal sex regulations[24]. Nonetheless, the presumption of atrazine as EDCs is still under scrutiny, whether it may or may not be the cause of potent effect on reproductive health[33, 34, 35]. Still, other herbicides present in the study sites (i.e., glyphosate, paraquat) may cause a potential effect on reproductive health, as previously observed on other frog species[36]. There are limited studies that focused on the estrogenic effect on female frogs[18] and in addition to our study, there is an observable herbicide impact on reproductive health of adult females' frogs living in contaminated sites.

Hepatosomatic index on adult male frogs from contaminated site (2.39 ± 0.091) was significantly higher than those from reference site (1.90 ± 0.041) (Student's *t*-test, $p < 0.05$, power of test [$\alpha = 0.05$] = 0.99). Similarly, HSI on adult female frogs from contaminated site (2.81 ± 0.070) was significantly higher than those from reference site (2.15 ± 0.133) (Mann Whitney rank sum test, $p < 0.05$, power of test [$\alpha = 0.05$] = 0.99). Based on the combined dataset of common frogs, it reveals similar trends showing significantly higher HSI on both sexes from contaminated site[31]. Studies on another frog species[28] and within the same frog species[18] also reveals similar results showing significantly higher HSI on both sexes from contaminated site. The heavier liver was regarded as the coping mechanism of the body to the unfavorable conditions[28] which also suggests xenobiotic contaminant accumulation[18]. It was reported that paraquat can reduce glutathione levels in the liver of fish[37]. Therefore, since liver serves as the main organ to detoxicate and accumulate contaminants, it must work harder to eliminate such compounds. In line with our study, there is an observable herbicide impact to the frog living in contaminated site by the changes of liver somatic indicating potential exposure of xenobiotics. The results summary of health parameters at organismal level (i.e., CF, GSI, HSI) is shown in Table 2 and Figure 3.

Table 2. Health parameters at organismal level including CF, GSI, and HSI (Mean ± SEM) of adult male and adult female rice frog *F. limnocharis* population from contaminated and reference site in Nan Province, Thailand, during the peak herbicide utilization period

Parameter	Sex	Contaminated site	Reference site	Power of test ($\alpha = 0.05$)[a]
Condition Factor	Adult male	104.69 ± 2.126 (N=23)	94.78 ± 2.163[*] (N=61)	0.67
	Adult female	105.18 ± 1.973 (N=30)	105.56 ± 2.266[ns] (N=40)	0.05
Gonadosomatic index	Adult male	0.30 ± 0.013 (N=20)	0.30 ± 0.015[ns] (N=35)	0.05
	Adult female	8.77 ± 0.439 (N=28)	6.98 ± 1.013[*] (N=14)	0.43
Hepatosomatic index	Adult male	2.39 ± 0.091 (N=20)	1.90 ± 0.041[*] (N=35)	0.99
	Adult female	2.81 ± 0.070 (N=28)	2.15 ± 0.133[*] (N=14)	0.99

[a]indicates the power of samples from both contaminated and reference sites (two-sample *t*-test, $\alpha = 0.05$)
[*]indicates significant difference between the contaminated and reference site (Mann-Whitney rank sum test/ Student's *t*-test, $p < 0.05$)
[ns]indicates no significant difference between the contaminated and reference site (Student's *t*-test, $p > 0.05$)

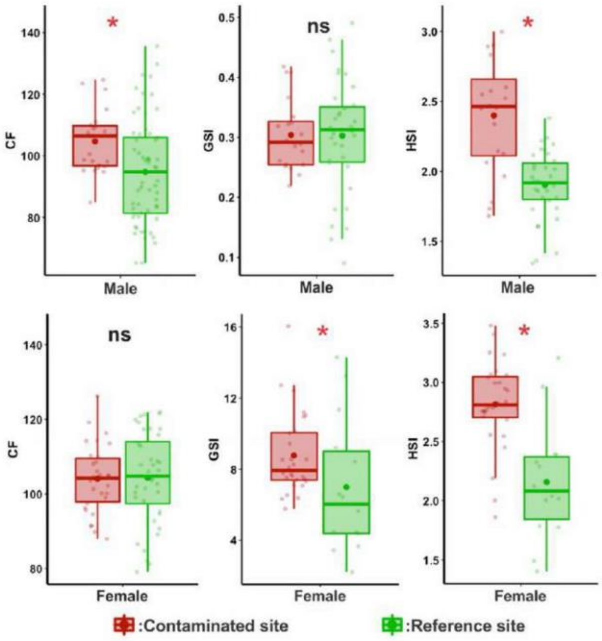

Figure 3. Boxplot of health parameters (CF, GSI, and HSI) of adult male and adult female rice frog *F. limnocharis* population from contaminated and reference site in Nan Province, Thailand, during the peak herbicide utilization period.

Size-frequency distribution

Both sexes show a higher means of SVL from contaminated site (38.30 ± 1.186) than those from reference site (36.74 ± 0.654). The size-frequency distribution shows significant site-related difference (two-sample Kolmogorov-Smirnov test, D = 0.4312, $p < 0.05$). Disproportionate distribution was observed prominently in contaminated sites, indicating the impact of herbicide contamination on the population structures. There are limited studies that directly focused on the population structure (i.e., size-frequency distribution). However, these irregular distributions might be due to reproductive failure, as one of the sexes could be dominant in the population which is likely caused by EDCs[38]. The global decline of amphibian population has also been linked to the immunotoxicity effect partly caused by agrochemicals, leading to the higher parasite load[39] in which certain life-stages (e.g., newly metamorphosing frogs) are found to be more sensitive[40]. To gains more insight into herbicide impacts on amphibian populations, additional studies (e.g., age structure, hatchling ratio, mortality rate) apart from size-frequency distribution should be widely encouraged. There is an observable herbicide impact on the size-frequency distribution of frogs living in contaminated sites showing disproportionate distribution, yet further investigations are needed to confirm this trend. The mean SVL and size-frequency distribution as a parameter on a population level is shown in Figure 4.

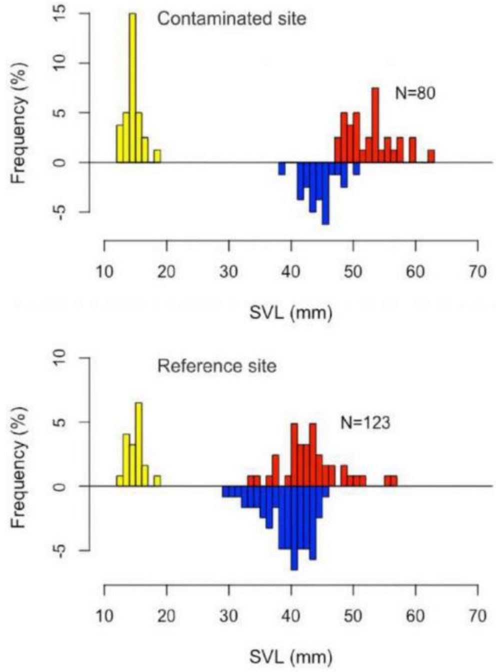

Figure 4. Size-frequency distribution (in percentage) of rice frog *F. limnocharis* population from contaminated and reference site in Nan Province, Thailand, during the peak herbicide utilization period. Yellow bar, red bar and blue bar correspond to froglets, female frogs and male frogs, respectively.

Conclusion:

Rice frogs plays important role in agroecosystem health by providing regulating services (controlling pest species), supporting ecosystem structure (soil burrowing, aquatic bioturbation), and nutrient cycling (food web)[41]. Due to their susceptibility and sensitivity to environmental stressors[9], amphibians are experiencing global decline[14], and by inference, humans may be losing the associated ecosystem services[41]. Our monitoring shows that herbicides could influence non-target organisms (i.e., rice frog) at organismal (CF, GSI, HSI) and population levels (growth pattern, size-frequency distribution) and may lead to subtle and perpetual changes in agroecosystem health. Further studies on contamination analysis and herbicide impacts on amphibian populations should be widely encouraged.

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References:

1. Xiao X, Boles S, Frolking S, Li C, Babu JY, Salas W, Moore III B. Remote Sens Environ. 2006;100(1):95–113.
2. Lam S, Pham G, Nguyen-Viet H. Int J Occup Med Environ Health. 2017;23(3):250–260.
3. Nicolopoulou-Stamati P, Maipas S, Kotampasi C, Stamatis P, Hens L. Front Public Health. 2016;4:148.
4. Mancini F, Woodcock BA, Isaac NJ. Curr Opin Environ Sustain. 2019;11:53–58.
5. Bossart GD. Vet Pathol. 2011;48(3):676–690.
6. Kitana N, Callard IP. J Environ Sci Health A. 2008;43(3):262–271.
7. Ali D, Nagpure NS, Kumar S, Kumar R, Kushwaha B, Lakra WS. Food Chem Toxicol. 2009;47(3):650–656.
8. Sparling DW, Bickham J, Cowman D, Fellers GM, Lacher T, Matson CW, McConnell L. Ecotoxicol. 2015;24(2):262–278.
9. Venturino A, Rosenbaum E, Caballero De Castro A, Anguiano OL, Gauna L, Fonovich De Schroeder T, Pechen De D'Angelo AM. Biomarkers. 2003;8(3–4):167–186.
10. Roy D. J Biosci. 2002;27(3):187.
11. Kloas W, Lutz I. J Chromatogr A. 2006;1130(1):16–27.
12. Burlibaşa L, Gavrilă L. Appl Ecol Environ Res. 2011;9(1):1–15.
13. Burggren WW, Warburton S. ILAR J. 2007;48(3):260–269.
14. Gahl MK, Pauli BD, Houlahan JE. Ecol Appl. 2011;21(7):2521–2529.
15. Laohaudomchok W, Nankongnab N, Siriruttanapruk S, Klaimala P, Lianchamroon W, Ousap P, Jatiket M, Kajitvichyanukul P, Kitana N, Siriwong W, Hemachudhah T, Satayavivad J, Robson M, Jaacks L, Barr DB, Kongtip P, Woskie S. Hum Ecol Risk Assess. 2020;27(5):1147–1169.
16. Maneein R, Khonsue W, Varanusupakul P, Noppadon K. Res J Chem Environ. 2011;15(2):1018–1023.
17. Thitiphuree T, Kitana J, Varanusupakul P, Kitana N. EnvironmentAsia. 2013;6(1):13–18.
18. Thammachoti P, Khonsue W, Kitana J, Varanusupakul P, Kitana N. J Environ Prot. 2012a;3(10):1403–1408.
19. Jantawongsri K, Thammachoti P, Kitana J, Khonsue W, Varanusupakul P, Kitana N. EnvironmentAsia. 2015;8(1):68–74.
20. Othman MS, Khonsue W, Kitana J, Thirakhupt K, Robson MG, Kitana N. Asian Herpetol Res. 2011;2(1):41–45.
21. Othman MS, Khonsue W, Kitana J, Thirakhupt K, Robson MG, Kitana N. Malaysian Journal of Health Sciences. 2016;14(2):57–64.
22. Brodeur JC, Suarez RP, Natale GS, Ronco AE, Zaccagnini ME. Ecotoxicol Environ. 2011;74(5):1370–1380.
23. Wickham H. Elegant graphics for data analysis. Media. 2009;35(211):1000–1007.
24. Hayes TB, Stuart AA, Mendoza M, Collins A, Noriega N, Vonk A, Johnston G, Liu R, Kpodzo D. Environ Health Perspect. 2006;114(Suppl 1):134–141.
25. Storrs SI, Semlitsch RD. Gen Comp Endocrinol. 2008;156(3):524–530.
26. Lenkowski JR, Sanchez-Bravo G, McLaughlin KA. J Environ Sci. 2010;22(9):1305–1308.
27. Hegde G, Krishnamurthy SV. EEC. 2014;9(1):69–76.
28. Zhelev ZM, Popgeorgiev GS, Mehterov NH. Bulg J Agric Sci. 2015;21(3):534–539.
29. Zhelev ZM, Tsonev SV, Arnaudova DN. Acta Zool. Bulg. 2017;8:169–176.

30. Schulte-Hostedde AI, Zinner B, Millar JS, Hickling GJ. Ecol. 2005;86(1):155–163.
31. Hegde G, Krishnamurthy SV, Berger G. Chem Ecol. 2019;35(5):397–407.
32. McKinlay R, Plant JA, Bell JN, Voulvoulis N. Environ Int. 2008;34(2):168–183.
33. Carr JA, Gentles A, Smith EE, Goleman WL, Urquidi LJ, Thuett K, Kendall RJ, Giesy JP, Gross TS, Solomon KR, Van Der Kraak G. Environ Toxicol Chem. 2003;22(2):396–405.
34. Murphy MB, Hecker M, Coady KK, Tompsett AR, Jones PD, Du Preez LH, Everson GJ, Solomon KR, Carr JA, Smith EE, Kendall RJ. Aquat Toxicol. 2006;76(3–4):230–245.
35. Hansen SP, Messer TL, Mittelstet AR. J Environ Manage. 2019;250:109424.
36. Quassinti L, Maccari E, Murri O, Bramucci M. Pestic Biochem Physiol. 2009;93(2):91–95.
37. Parvez S, Raisuddin S. Arch Environ Contam Toxicol. 2006;50(3):392–397.
38. Lambert MR, Giller GS, Barber LB, Fitzgerald KC, Skelly DK. PNAS. 2015;112(38):11881–11886.
39. Köhler HR, Triebskorn R. Science. 2013;341(6147):759–765.
40. Rollins-Smith LA, Ramsey JP, Pask JD, Reinert LK, Woodhams DC. Integr Comp Biol. 2011;51(4):552–562.
41. Hocking DJ, Babbitt KJ. Herpetol. Conserv. Biol. 2014;9(1):1–17.

REFERENCES

Agas, D., Sabbieti, M. G., and Marchetti, L. 2013. Endocrine disruptors and bone metabolism. Archives of Toxicology, 87(4): 735–751.

Agency for Toxic Substances and Diseases Registry. 2003. Toxicological Profile for Atrazine. Atlanta, USA: Public Health Services.

Agency for Toxic Substances and Diseases Registry. 2020. Toxicological Profile for Glyphosate. Atlanta, USA: Public Health Services.

Alferness, P. L. and Iwata, Y. 1994. Determination of glyphosate and (Aminomethyl) phosphonic acid in soil, plant and animal matrixes, and water by capillary gas chromatography with mass-selective detection. Journal of Agricultural and Food Chemistry, 42(12): 2751–2759.

Altherr, S., Goyenechea, A., and Schubert, D. J. 2011. The International Trade in Frogs' Legs and Its Ecological Impact. Munich, Germany; Washington D.C., USA: Pro Wildlife, Defenders of Wildlife and Animal Welfare Institute.

AmphibiaWeb. 2021. *Fejervarya limnocharis*: Alpine Cricket Frog [Online]. Available from: https://amphibiaweb.org/species/4770 [9 April 2021].

Babalola, O. O., Truter, J. C., and van Wyk, J. H. 2019. Mortality, teratogenicity and growth inhibition of three glyphosate formulations using frog embryo teratogenesis assay-*Xenopus*. Journal of Applied Toxicology, 39(9): 1257–1266.

Bach, N. C., Marino, D. J., Natale, G. S., and Somoza, G. M. 2018. Effects of glyphosate and its commercial formulation, Roundup® Ultramax, on liver histology of tadpoles of the neotropical frog, *Leptodactylus latrans* (Amphibia: Anura). Chemosphere, 202: 289–297.

Bach, N. C., Natale, G. S., Somoza, G. M., and Ronco, A. E. 2016. Effect on the growth and development and induction of abnormalities by a glyphosate commercial formulation and its active ingredient during two developmental stages of the South-American creole frog, *Leptodactylus latrans*. Environmental Science and Pollution Research, 23(23): 23959–23971.

Baker, N. J., Bancroft, B. A., and Garcia, T. S. 2013. A meta-analysis of the effects of pesticides and fertilizers on survival and growth of amphibians. Science of the Total Environment, 449: 150–156.

Barchanska, H., Sajdak, M., Szczypka, K., Swientek, A., Tworek, M., and Kurek, M. 2017. Atrazine, triketone herbicides, and their degradation products in sediment, soil and surface water samples in Poland. Environmental Science and Pollution Research, 24(1): 644–658.

Barus, V., Jarkovsky, J., and Prokes, M. 2007. *Philometra ovata* (Nematoda: Philometroidea): A potential sentinel species of heavy metal accumulation. Parasitology Research, 100(5): 929–933.

Birch, M. 1993. Reregistration Eligibility Decision (RED) Glyphosate. (EPA-738-R-93-014; 4-70-90, 1970). Washington D.C., USA: U.S. Government Printing Office.

Blaustein, A. R. and Wake, D. B. 1990. Declining amphibian populations: A global phenomenon? Trends in Ecology & Evolution, 5(7): 203–204.

Boone, M. D. 2005. Juvenile frogs compensate for small metamorph size with terrestrial growth: Overcoming the effects of larval density and insecticide exposure. Journal of Herpetology, 39(3): 416–423.

Bossart, G. D. 2011. Marine mammals as sentinel species for oceans and human health. Veterinary Pathology, 48(3): 676–690.

Brown, P., Charlton, A., Cuthbert, M., Barnett, L., Ross, L., Green, M., Gillies, L., Shaw, K., and Fletcher, M. 1996. Identification of pesticide poisoning in wildlife. Journal of Chromatography A, 754(1): 463–478.

Brühl, C. A., Pieper, S., and Weber, B. 2011. Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. Environmental Toxicology and Chemistry, 30(11): 2465–2472.

Burggren, W. W. and Warburton, S. 2007. Amphibians as animal models for laboratory research in physiology. ILAR Journal, 48(3): 260–269.

Burlibaşa, L. and Gavrilă, L. 2011. Amphibians as model organisms for study environmental genotoxicity. Applied Ecology and Environmental Research, 9(1): 1–15.

Carr, J. A., Gentles, A., Smith, E. E., Goleman, W. L., Urquidi, L. J., Thuett, K., Kendall, R. J., Giesy, J. P., Gross, T. S., and Solomon, K. R. 2003. Response of larval *Xenopus* *laevis* to atrazine: Assessment of growth, metamorphosis, and gonadal and laryngeal morphology. Environmental Toxicology and Chemistry: An International Journal, 22(2): 396–405.

Carvalho, F. P. 2017. Pesticides, environment, and food safety. Food and Energy Security, 6(2): 48–60.

Chan-ard, T. 2003. A Photographic Guide to Amphibians in Thailand. Bangkok, Thailand: Darnsutha Press Company, pp. 176.

Chanpong, P. 2008. The Record of Agrochemicals, Chemical Fertilizer and Organic Fertilizer Utilization at Nan Province in 2008 (in Thai). Nan, Thailand: Nan Provincial Agricultural Extension Office.

Codex Alimentarius. 2006. Pesticide Index [Online]. Available from: http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/pestres/pesticides/en [3 January 2021].

Costa, R. N. and Nomura, F. 2016. Measuring the impacts of Roundup Original® on fluctuating asymmetry and mortality in a neotropical tadpole. Hydrobiologia, 765(1): 85–96.

Crawshaw, G. J. and Weinkle, T. K. 2000. Clinical and pathological aspects of the amphibian liver. Seminars in Avian and Exotic Pet Medicine, 9(3): 165–173.

Davidson, C. 2004. Declining downwind: Amphibian population declines in California and historical pesticide use. Ecological Applications, 14(6): 1892–1902.

de Brito Rodrigues, L., Gonçalves Costa, G., Lundgren Thá, E., da Silva, L. R., de Oliveira, R., Morais Leme, D., Cestari, M. M., Koppe Grisolia, C., Campos Valadares, M., and de Oliveira, G. A. R. 2019. Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 842: 94–101.

Devine, M., Duke, S. O., and Fedtke, C. 1992. Physiology of Herbicide Action. New Jersey, USA: PTR Prentice Hall, pp. 441.

di Rosa, I., Clarioni, R., Hotz, H., Pascolini, R., Simoncelli, F., Fagotti, A., Morosi, L., Pellegrino, R., and Guex, G. D. 2005. Bioaccumulation of organochlorine pesticides in frogs of the *Rana esculenta* complex in central Italy. Amphibia-

Reptilia, 26(1): 93–104.

Dial, N. A. and Bauer, C. A. 1984. Teratogenic and lethal effects of paraquat on developing frog embryos (*Rana pipiens*). Bulletin of Environmental Contamination and Toxicology, 33(1): 592–597.

Didde, R. D. and Rivera, G. 2019. Patterns of fluctuating asymmetry in the limbs of anurans. Journal of Morphology, 280(4): 587–592.

Duellman, W. E. and Trueb, L. 1994. Biology of Amphibians. Baltimore, USA: Johns Hopkins University Press, pp. 670.

Eftekhari, A., Hasanzadeh, A., Khalilov, R., Hosainzadegan, H., Ahmadian, E., and Eghbal, M. A. 2020. Hepatoprotective role of berberine against paraquat-induced liver toxicity in rat. Environmental Science and Pollution Research, 27(5): 4969–4975.

Eisemberg, C. C. and Bertoluci, J. 2016. Fluctuating asymmetry in populations of the South American frog *Physalaemus cuvieri* (Leptodactylidae) in areas with different degrees of disturbance. Journal of Natural History, 50(23–24): 1503–1511.

Eterovick, P. C., Sloss, B. L., Scalzo, J. A., and Alford, R. A. 2016. Isolated frogs in a crowded world: Effects of human-caused habitat loss on frog heterozygosity and fluctuating asymmetry. Biological Conservation, 195: 52–59.

Fan, W. Q., Yanase, T., Morinaga, H., Gondo, S., Okabe, T., Nomura, M., Komatsu, T., Morohashi, K. I., Hayes, T. B., and Takayanagi, R. 2007. Atrazine-induced aromatase expression is SF-1 dependent: Implications for endocrine disruption in wildlife and reproductive cancers in humans. Environmental Health Perspectives, 115(5): 720–727.

Folmar, L. C., Sanders, H., and Julin, A. 1979. Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates. Archives of Environmental Contamination and Toxicology, 8(3): 269–278.

Frost, D. R. 2021. Amphibian species of the world: An online reference. version 6.1. Available from: https://amphibiansoftheworld.amnh.org/index.php[3 January 2021]

Gahl, M. K., Pauli, B. D., and Houlahan, J. E. 2011. Effects of chytrid fungus and a glyphosate-based herbicide on survival and growth of wood frogs (*Lithobates sylvaticus*). Ecological Applications, 21(7): 2521–2529.

Giesy, J. P., Dobson, S., and Solomon, K. R. 2000. Ecotoxicological risk assessment for Roundup® herbicide. In G. W. Ware (ed.), Reviews of Environmental Contamination and Toxicology, pp. 35–120. New York, USA: Springer.

Gilbert, S. F. and Barresi, J. F. 2016. Developmental Biology 11th ed. Massachusetts, USA: Sinauer Associates Inc., pp. 810.

Golden, N. H. and Rattner, B. A. 2003. Ranking terrestrial vertebrate species for utility in biomonitoring and vulnerability to environmental contaminants. Reviews of Environmental Contamination and Toxicology, 176: 67–136.

Gondim, P. D. M., Rodrigues, J. F. M., and Cascon, P. 2020. Fluctuating asymmetry and organosomatic indices in anuran populations in agricultural environments in semi-arid Brazil. Herpetological Conservation and Biology, 15(2): 354–366.

Government of Thailand. 2020. Notification B.E. 2563 (2020): List of Hazardous Substances (Volume 6). Bangkok, Thailand: The Royal Gazette.

Govindarajulu, P. P. 2008. Literature Review of Impacts of Glyphosate Herbicide on Amphibians: What Risks Can the Silvicultural Use of This Herbicide Pose for Amphibians in BC? Victoria, British Columbia: Ecosystems Branch, Ministry of Environment, pp. 79.

Guillot, H., Boissinot, A., Angelier, F., Lourdais, O., Bonnet, X., and Brischoux, F. 2016. Landscape influences the morphology of male common toads (*Bufo bufo*). Agriculture, Ecosystems & Environment, 233: 106–110.

Haley, T. J. 1979. Review of the toxicology of paraquat (1, 1′-dimethyl-4, 4′-bipyridinium chloride). Clinical Toxicology, 14(1): 1–46.

Hansen, B. H., Rømma, S., Garmo, Ø. A., Olsvik, P. A., and Andersen, R. A. 2006. Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 143(3): 263–274.

Hanson, M. L., Solomon, K. R., van der Kraak, G. J., and Brian, R. A. 2019. Effects of atrazine on fish, amphibians, and reptiles: Update of the analysis based on quantitative weight of evidence. Critical Reviews in Toxicology, 49(8): 670–709.

Hasan, M., Islam, M. M., Khan, M. M. R., Igawa, T., Alam, M. S., Djong, H. T., Kurniawan, N., Joshy, H., Sen, Y. H., and Belabut, D. M. 2014. Genetic divergences of South and Southeast Asian frogs: A case study of several taxa based on 16S ribosomal RNA gene data with notes on the generic name *Fejervarya*. Turkish Journal of Zoology, 38(4): 389–411.

Hayes, T. B., Anderson, L. L., Beasley, V. R., De Solla, S. R., Iguchi, T., Ingraham, H., Kestemont, P., Kniewald, J., Kniewald, Z., and Langlois, V. S. 2011. Demasculinization and feminization of male gonads by atrazine: Consistent effects across vertebrate classes. The Journal of Steroid Biochemistry and Molecular Biology, 127(1-2): 64–73.

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A., and Vonk, A. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences, 99(8): 5476–5480.

Hayes, T. B., Khoury, V., Narayan, A., Nazir, M., Park, A., Brown, T., Adame, L., Chan, E., Buchholz, D., and Stueve, T. 2010. Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proceedings of the National Academy of Sciences, 107(10): 4612–4617.

Hayes, T. B., Stuart, A. A., Mendoza, M., Collins, A., Noriega, N., Vonk, A., Johnston, G., Liu, R., and Kpodzo, D. 2006. Characterization of atrazine-induced gonadal malformations in African clawed frogs (*Xenopus laevis*) and comparisons with effects of an androgen antagonist (cyproterone acetate) and exogenous estrogen (17β-estradiol): Support for the demasculinization/feminization hypothesis. Environmental Health Perspectives, 114(Suppl 1): 134–141.

Health Canada's Pesticides & Pest Management. 2011. Maximum Residue Limits for Pesticides [Online]. Available from: https://pr-rp.hc-sc.gc.ca/mrl-lrm/results-eng.php [3 January 2021].

Hegde, G. and Krishnamurthy, S. V. 2014. Analysis of health status of the frog *Fejervarya* *limnocharis* (Anura: Ranidae) living in rice paddy fields of Western Ghats, using body condition factor and AChE content. Ecotoxicology and Environmental Contamination, 9(1): 69–76.

Hegde, G., Krishnamurthy, S. V., and Berger, G. 2019. Common frogs response to agrochemicals contamination in coffee plantations, Western Ghats, India. Chemistry and Ecology, 35(5): 397–407.

Hirai, T. and Matsui, M. 2001. Diet composition of the Indian rice frog, *Rana limnocharis*, in rice fields of central Japan. Current Herpetology, 20(2): 97–103.

Hoskins, T. D. and Boone, M. D. 2018. Atrazine feminizes sex ratio in Blanchard's cricket frogs (*Acris blanchardi*) at concentrations as low as 0.1 μg/L. Environmental Toxicology and Chemistry, 37(2): 427–435.

Howard, P. H. 1991. Handbook of Environmental Degradation Rates 1st ed. Boca Raton, USA: CRC Press, pp. 776.

Howe, C. M., Berrill, M., Pauli, B. D., Helbing, C. C., Werry, K., and Veldhoen, N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry: An International Journal, 23(8): 1928–1938.

Hu, E. and Cheng, H. 2013. Rapid extraction and determination of atrazine and its degradation products from microporous mineral sorbents using microwave-assisted solvent extraction followed by ultra-HPLC-MS/MS. Microchimica Acta, 180(7–8): 703–710.

Intamat, S., Phoonaploy, U., Sriuttha, M., Patawang, I., Tanomtong, A., and Neeratanaphan, L. 2016. Cytotoxic evaluation of rice field frogs (*Fejervarya limnocharis*) from gold mine area with arsenic contamination. The Nucleus, 59(3): 181–189.

Isani, G., Andreani, G., Cocchioni, F., Fedeli, D., Carpene, E., and Falcioni, G. 2009. Cadmium accumulation and biochemical responses in *Sparus aurata* following sub-lethal Cd exposure. Ecotoxicology and Environmental Safety, 72(1): 224–230.

Iskandar, D. T. 1998. The Amphibians of Java and Bali. Bogor, Indonesia: Research and

Development Centre for Biology-LIPI, pp. 117.

Islam, M. M., Kurose, N., Khan, M. R., Nishizawa, T., Kuramoto, M., Alam, M. S., Hasan, M., Kurniawan, N., Nishioka, M., and Sumida, M. 2008. Genetic divergence and reproductive isolation in the genus *Fejervarya* (Amphibia: Anura) from Bangladesh inferred from morphological observations, crossing experiments, and molecular analyses. Zoological Science, 25(11): 1084–1105.

Jacomini, A. E., Bonato, P. S., and Avelar, W. E. P. 2003. HPLC method for the analysis of atrazine in freshwater bivalves. Journal of Liquid Chromatography & Related Technologies, 26(12): 1885–1894.

Jantawongsri, K., Thammachoti, P., Kitana, J., Khonsue, W., Varanusupakul, P., and Kitana, N. 2015. Altered immune response of the rice frog *Fejervarya limnocharis* living in agricultural area with intensive herbicide utilization at Nan Province, Thailand. EnvironmentAsia, 8(1): 68–74.

Jilani, M. J., Rais, M., Asadi, M. A., and Mahmood, T. 2018. Comparison of morphometric and gravimetric measurements of common skittering frog (*Euphlyctis cyanophlyctis*) from paddy fields and urban wetlands. Journal of King Saud University-Science, 30(3): 404–411.

Kaiser, J. 2001. Bioindicators and Biomarkers of Environmental Pollution and Risk Assessment. Enfield, USA: Science Publishers Inc., pp. 204.

Kim, J. W. and Kim, D. S. 2020. Paraquat: Toxicology and impacts of its ban on human health and agriculture. Weed Science, 68(3): 208–213.

Kitana, N., Won, S. J., and Callard, I. P. 2007. Reproductive deficits in male freshwater turtle *Chrysemys picta* from Cape Cod, Massachusetts. Biology of Reproduction, 76(3): 346–352.

Kloas, W. and Lutz, I. 2006. Amphibians as model to study endocrine disrupters. Journal of Chromatography A, 1130(1): 16–27.

Köhler, H. R. and Triebskorn, R. 2013. Wildlife ecotoxicology of pesticides: Can we track effects to the population level and beyond? Science, 341(6147): 759–765.

Krishnamurthy, S. V., Meenakumari, D., Gurushankara, H. P., and Vasudev, V. 2008. Nitrate-induced morphological anomalies in the tadpoles of *Nyctibatrachus major* and *Fejervarya limnocharis* (Anura: Ranidae). Turkish Journal of Zoology, 32(3): 239–244.

Kusrini, M. D. 2005. Edible Frog Harvesting in Indonesia: Evaluating Its Impact and Ecological Context. Doctoral's Dissertation. James Cook University, Australia, pp. 256.

Kusrini, M. D. 2019. Metode Survei dan Penelitian Herpetofauna (in Bahasa Indonesia). Bogor, Indonesia: PT Penerbit IPB Press.

Lajmanovich, R., Izaguirre, M., and Casco, V. 1998. Paraquat tolerance and alteration of internal gill structure of *Scinax nasica* tadpoles (Anura: Hylidae). Archives of Environmental Contamination and Toxicology, 34(4): 364–369.

Lam, S., Pham, G., and Nguyen-Viet, H. 2017. Emerging health risks from agricultural intensification in Southeast Asia: A systematic review. International Journal of Occupational and Environmental Health, 23(3): 250–260.

Lambert, M. R., Giller, G. S., Barber, L. B., Fitzgerald, K. C., and Skelly, D. K. 2015. Suburbanization, estrogen contamination, and sex ratio in wild amphibian populations. Proceedings of the National Academy of Sciences, 112(38): 11881–11886.

Lanctot, C., Navarro-Martín, L., Robertson, C., Park, B., Jackman, P., Pauli, B. D., and Trudeau, V. L. 2014. Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frog (*Lithobates sylvaticus*) tadpoles. II: Agriculturally relevant exposures to Roundup WeatherMax® and Vision® under laboratory conditions. Aquatic Toxicology, 154: 291–303.

Land Development Department. 2018a. Land used information [Online]. Available from: http://www1.ldd.go.th/WEB_OLP/Lu_61/Lu61_N/NAN61.htm [3 January 2021].

Land Development Department. 2018b. Land used map [Online]. Available from: http://www1.ldd.go.th/WEB_OLP/Lu_61/Lu61_N/map61/A4_NAN61.jpg [3 January 2021].

Laohaudomchok, W., Nankongnab, N., Siriruttanapruk, S., Klaimala, P., Lianchamroon, W., Ousap, P., Jatiket, M., Kajitvichyanukul, P., Kitana, N., and Siriwong, W. 2020. Pesticide use in Thailand: Current situation, health risks, and gaps in research and policy. Human and Ecological Risk Assessment: An International Journal, 27(5): 1147–1169.

Lauck, B. 2006. Fluctuating asymmetry of the frog *Crinia signifera* in response to logging. Wildlife Research, 33(4): 313–320.

Lenkowski, J. R., Sanchez-Bravo, G., and McLaughlin, K. A. 2010. Low concentrations of atrazine, glyphosate, 2, 4-dichlorophenoxyacetic acid, and triadimefon exposures have diverse effects on *Xenopus laevis* organ morphogenesis. Journal of Environmental Sciences, 22(9): 1305–1308.

Leong, W. H., Teh, S. Y., Hossain, M. M., Nadarajaw, T., Zabidi Hussin, Z., Chin, S. Y., Lai, K. S., and Lim, S. H. E. 2020. Application, monitoring and adverse effects in pesticide use: The importance of reinforcement of Good Agricultural Practices (GAPs). Journal of Environmental Management, 260: 109987.

Liao, W. B., Lu, X., Shen, Y. W., and Hu, J. C. 2011. Age structure and body size of two populations of the rice frog *Rana limnocharis* from different altitudes. Italian Journal of Zoology, 78(2): 215–221.

Lindquist, N., Larsson, B., and Lyden-Sokolowski, A. 1988. Autoradiography of [14C] paraquat or [14C] diquat in frogs and mice: Accumulation in neuromelanin. Neuroscience Letters, 93(1): 1–6.

Liu, J., Wang, M., Yang, L., Rahman, S., and Sriboonchitta, S. 2020. Agricultural productivity growth and its determinants in South and Southeast Asian countries. Sustainability, 12(12): 4981.

Mambro, A. D. 2015. Farming in Mountainous Areas: A Fragile Balance [Online]. Available from: http://www.euractiv.com/section/agriculture-food/news/farming-in-mountainous-areas-a-fragile-balance [3 January 2021].

Maneein, R. 2012. Biologic Responses of Rice Field Crab *Esanthelphusa nani* (Naiyanetr, 1984) to Herbicides in Paddy Fields, Nan Province. Master's Thesis. Chulalongkorn University, Thailand, pp. 114.

Mann, R. M., Hyne, R. V., Choung, C. B., and Wilson, S. P. 2009. Amphibians and agricultural chemicals: Review of the risks in a complex environment. Environmental Pollution, 157(11): 2903–2927.

Meza-Joya, F. L., Ramírez-Pinilla, M. P., and Fuentes-Lorenzo, J. L. 2013. Toxic, cytotoxic, and genotoxic effects of a glyphosate formulation (Roundup® SL–Cosmoflux®

411F) in the direct-developing frog *Eleutherodactylus johnstonei*. Environmental and Molecular Mutagenesis, 54(5): 362–373.

Mikó, Z., Ujszegi, J., Gál, Z., and Hettyey, A. 2017. Effects of a glyphosate-based herbicide and predation threat on the behaviour of agile frog tadpoles. Ecotoxicology and Environmental Safety, 140: 96–102.

Moore, J. K., Braymer, H. D., and Larson, A. D. 1983. Isolation of a *Pseudomonas* sp. which utilizes the phosphonate herbicide glyphosate. Applied and Environmental Microbiology, 46(2): 316–320.

Murphy, M., Hecker, M., Coady, K., Tompsett, A., Jones, P., du Preez, L. H., Everson, G., Solomon, K. R., Carr, J., and Smith, E. 2006. Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. Aquatic Toxicology, 76(3-4): 230–245.

Nataraj, M. B. and Krishnamurthy, S. V. 2013. Exposure of tadpoles of *Fejervarya limnocharis* (Anura: Ranidae) to combinations of carbaryl and cypermethrin. Toxicological & Environmental Chemistry, 95(8): 1408–1415.

National Center for Biotechnology Information. 2021a. PubChem Compound Summary for CID 2256, Atrazine. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Atrazine[4 February 2021]

National Center for Biotechnology Information. 2021b. PubChem Compound Summary for CID 3496, Glyphosate. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Glyphosate[4 February 2021]

National Center for Biotechnology Information. 2021c. PubChem Compound Summary for CID 15939, Paraquat. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Paraquat[4 February 2021]

National Research Council. 1991. Animals as Sentinels of Environmental Health Hazards. Washington D. C., USA: National Academy Press, pp. 176.

Neang, T. 2010. An Investigation into Frog Consumption and Trade in Cambodia vol. 25. Phnom Penh, Cambodia: Fauna & Flora International Cambodia Programme, pp. 24.

Norris, D. O., Camp, J. M., Maldonado, T. A., and Woodling, J. D. 2000. Some aspects of hepatic function in feral brown trout, *Salmo trutta*, living in metal contaminated water. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 127(1): 71–78.

Osano, O., Oladimeji, A., Kraak, M., and Admiraal, W. 2002. Teratogenic effects of amitraz, 2, 4-dimethylaniline, and paraquat on developing frog (*Xenopus*) embryos. Archives of Environmental Contamination and Toxicology, 43(1): 42–49.

Othman, M. S. 2009. Using the Rice Frog (*Fejervarya limnocharis*) as a Sentinel Species for Cadmium Contamination in Tak Province, Thailand. Doctoral's Dissertation. Chulalongkorn University, Thailand, pp. 203.

Othman, M. S., Khonsue, W., Kitana, J., Thirakhupt, K., Robson, M., Borjan, M., and Kitana, N. 2012. Hepatic metallothionein and glutathione-S-transferase responses in two populations of rice frogs, *Fejervarya limnocharis*, naturally exposed to different environmental cadmium levels. Bulletin of Environmental Contamination and Toxicology, 89(2): 225–228.

Othman, M. S., Khonsue, W., Kitana, J., Thirakhupt, K., Robson, M. G., and Kitana, N. 2009. Cadmium accumulation in two populations of rice frogs (*Fejervarya limnocharis*) naturally exposed to different environmental cadmium levels. Bulletin of Environmental Contamination and Toxicology, 83(5): 703–707.

Othman, M. S., Khonsue, W., Kitana, J., Thirakhupt, K., Robson, M. G., and Kitana, N. 2011. Reproductive mode of *Fejervarya limnocharis* (Anura: Ranidae) caught from Mae Sot, Thailand based on its gonadosomatic indices. Asian Herpetological Research, 2(1): 41–45.

Othman, M. S., Khonsue, W., Kitana, J., Thirakhupt, K., Robson, M. G., and Kitana, N. 2016. Morphometric and gravimetric indices of two populations of rice frog (*Fejervarya limnocharis*) naturally exposed to different environmental cadmium levels. Jurnal Sains Kesihatan Malaysia, 14(2): 57–64.

Palmer, A. R. 1994. Fluctuating Asymmetry Analyses: A Primer. In T. A. Markow (ed.), Developmental instability: Its origins and evolutionary implications, pp. 335–364. Dordrecht, Netherlands: Springer.

Palmer, A. R. and Strobeck, C. 1986. Fluctuating asymmetry: Measurement, analysis, patterns. Annual Review of Ecology and Systematics, 17(1): 391–421.

Palmer, A. R. and Strobeck, C. 1992. Fluctuating asymmetry as a measure of developmental stability: Implications of non-normal distributions and power of statistical tests. Acta Zoologica Fennica, 191: 57–72.

Palmer, A. R. and Strobeck, C. 2003. Fluctuating Asymmetry Analyses Revisited. In M. Polak (ed.), Developmental Instability: Causes and Consequences, pp. 279–319. Oxford, UK: Oxford University Press.

Pancharatna, K. and Deshpande, S. A. 2003. Skeletochronological data on age, body size and mass in the Indian cricket frog *Limnonectes limnocharis* (Boie, 1835). Herpetozoa, 16(1/2): 41–50.

Parvez, S. and Raisuddin, S. 2006. Effects of paraquat on the freshwater fish *Channa punctata* (Bloch): Non-enzymatic antioxidants as biomarkers of exposure. Archives of Environmental Contamination and Toxicology, 50(3): 392–397.

Patarasiriwong, V. 2016. Study and Development of Risk Management Guidelines from Organophospahates in the Upper Northern Areas with Participatory Research Process, Year-1: Chiang Rai and Nan Provinces (in Thai). Thailand: Environmental Research and Training Center, Department of Environmental Quality Promotion, Ministry of Natural Resources and Environment.

Petit, V., Cabridenc, R., Swannell, R., and Sokhi, R. 1995. Review of strategies for modelling the environmental fate of pesticides discharged into riverine systems. Environment International, 21(2): 167–176.

Phadmacanty, N. L. P. R., Hamidy, A., and Semiadi, G. 2019. On skeletochronology of Asian grass frog *Fejervarya limnocharis* (Gravenhorst, 1829) from Java to support management conservation. Treubia, 45: 1–10.

Quassinti, L., Maccari, E., Murri, O., and Bramucci, M. 2009. Effects of paraquat and glyphosate on steroidogenesis in gonads of the frog *Rana esculenta in vitro*. Pesticide Biochemistry and Physiology, 93(2): 91–95.

Quick, M., Dyson, D., and Holliman, A. 1990. Acute and sub-acute paraquat poisoning in a pack of foxhounds. Journal-Forensic Science Society, 30(6): 371–376.

R Core Team. (2017). R: A Language and Environment for Statistical Computing (version 3.4.1). Vienna, Austria: R Foundation for Statistical Computing.

Relyea, R. A. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecological Applications, 15(2): 618–627.

Riaño, C., Ortiz-Ruiz, M., Pinto-Sánchez, N. R., and Gómez-Ramírez, E. 2020. Effect of glyphosate (Roundup Active®) on liver of tadpoles of the Colombian endemic frog *Dendropsophus molitor* (Amphibia: Anura). Chemosphere, 250(126287): 1–7.

Rie, M. T., Kitana, N., Lendas, K. A., Won, S. J., and Callard, I. P. 2005. Reproductive endocrine disruption in a sentinel species (*Chrysemys picta*) on Cape Cod, Massachusetts. Archives of Environmental Contamination and Toxicology, 48(2): 217–224.

Rimayi, C., Odusanya, D., Weiss, J. M., de Boer, J., Chimuka, L., and Mbajiorgu, F. 2018. Effects of environmentally relevant sub-chronic atrazine concentrations on African clawed frog (*Xenopus laevis*) survival, growth and male gonad development. Aquatic Toxicology, 199: 1–11.

Roberts, T. R. 1996. Assessing the environmental fate of agrochemicals. Journal of Environmental Science & Health Part B, 31(3): 325–335.

Rollins-Smith, L. A., Rice, C. D., and Grasman, K. A. 2006. Amphibian, Fish, and Bird Immunotoxicology. In R. V. House, R. Luebke, & I. Kimber (eds.), Immunotoxicology and Immunopharmacology, pp. 409–426. Boca Raton, USA: CRC Press.

Ronald, E. 1990. Paraquat Hazards to Fish, Wildlife, and Invertebrates: A Synoptic Review. Laurel, Maryland: Patuxent Wildlife Research Center.

Roy, D. 2002. Amphibians as environmental sentinels. Journal of Biosciences, 27(3): 187–188.

Rujivanarom, P. (16 May 2018). Farm Chemicals ‘Pose a Threat to Public Health’. The Nation Thailand. Available from: https://www.nationthailand.com/in-focus/30345563

Ruksachat, N., Chukanhom, K., Tengjaroenkul, B., and Neeratanaphan, L. 2021. The first report of the effect of glyphosate on chromosomal aberrations in the threatened Sapgreen stream frog (*Sylvirana nigrovittata*) *in vivo*. International Journal of Environmental Studies, 78(2): 319–331.

Scribner, E. A., Thurman, E., and Zimmerman, L. R. 2000. Analysis of selected herbicide metabolites in surface and ground water of the United States. Science of the Total Environment, 248(2–3): 157–167.

Singh, S. N. and Jauhari, N. 2017. Degradation of Atrazine by Plants and Microbes. In S. Singh (ed.), Microbe-Induced Degradation of Pesticides, pp. 213–225. Cham, USA: Springer.

Söderman, F., van Dongen, S., Pakkasmaa, S., and Merilä, J. 2007. Environmental stress increases skeletal fluctuating asymmetry in the moor frog *Rana arvalis*. Oecologia, 151(4): 593–604.

Solomon, K. R., Carr, J. A., du Preez, L. H., Giesy, J. P., Kendall, R. J., Smith, E. E., and van der Kraak, G. J. 2008. Effects of atrazine on fish, amphibians, and aquatic reptiles: A critical review. Critical Reviews in Toxicology, 38(9): 721–772.

Sparling, D. W., Fellers, G. M., and McConnell, L. L. 2001. Pesticides and amphibian population declines in California, USA. Environmental Toxicology and Chemistry: An International Journal, 20(7): 1591–1595.

Stahl, R. G. 1997. Can mammalian and non-mammalian "sentinel species"; data be used to evaluate the human health implications of environmental contaminants? Human and Ecological Risk Assessment: An International Journal, 3(3): 329–335.

Sumida, M., Kotaki, M., Islam, M. M., Djong, T. H., Igawa, T., Kondo, Y., Matsui, M., Khonsue, W., and Nishioka, M. 2007. Evolutionary relationships and reproductive isolating mechanisms in the rice frog (*Fejervarya limnocharis*) species complex from Sri Lanka, Thailand, Taiwan and Japan, inferred from mtDNA gene sequences, allozymes, and crossing experiments. Zoological Science, 24(6): 547–562.

Tanakasempipat, P. (1 June 2020). Thailand's Chemical Pesticide Ban Troubles Farmers, Industries. Reuters. Available from: https://www.reuters.com/article/us-thailand-chemicals-idUSKBN2382IC

Tavalieri, Y., Galoppo, G., Canesini, G., Luque, E., and Muñoz de Toro, M. 2020. Effects of agricultural pesticides on the reproductive system of aquatic wildlife species, with crocodilians as sentinel species. Molecular and Cellular Endocrinology, 518(110918): 1–15.

Taylor, B., Skelly, D., Demarchis, L. K., Slade, M. D., Galusha, D., and Rabinowitz, P. M. 2005. Proximity to pollution sources and risk of amphibian limb malformation. Environmental Health Perspectives, 113(11): 1497–1501.

Taylor, M. (5 August 2020). Farmers Call on Thai Government to Overturn Ban on Pesticide Use. Thaiger. Available from: https://thethaiger.com/hot-news/environment/farmers-call-on-thai-government-to-overturn-ban-on-pesticide-use

Thammachoti, P. 2012. Influence of Herbicides on Morphology and Population of Rice Frog *Fejervarya limnocharis* (Gravenhost, 1829) in Paddy Fields, Nan Province. Master's Thesis. Chulalongkorn University, Thailand, pp. 171.

Thammachoti, P., Khonsue, W., Kitana, J., Varanusupakul, P., and Kitana, N. 2012. Morphometric and gravimetric parameters of the rice frog *Fejervarya limnocharis* living in areas with different agricultural activity. Journal of Environmental Protection, 3(10): 1403–1408.

Thanomsit, C., Saowakoon, S., Wattanakornsiri, A., Nanuam, J., Prasatkaew, W., Nanthanawat, P., Mongkolvai, P., and Chalorcharoenying, W. 2020. The Glyphosate (Roundup): Fate in aquatic environment, adverse effect and toxicity assessment in aquatic organisms. Naresuan University Journal: Science and Technology (NUJST), 28(1): 65–81.

Thitiphuree, T. 2012. Association between Herbicide Contamination and Reproductive Effects in Freshwater Mussel *Uniandra contradens* in Agricultural Areas, Nan Province. Master' Thesis. Chulalongkorn University, Thailand, pp. 157.

Toda, M., Matsui, M., Nishida, M., and Ota, H. 1998. Genetic divergence among Southeast and East Asian populations of *Rana limnocharis* (Amphibia: Anura), with special reference to sympatric cryptic species in Java. Zoological Science, 15(4): 607–613.

U.S. Environmental Protection Agency. 1993a. Reregistration Eligibility Decision (RED) Document Glyphosate. List A Case 0178. Washington D.C., USA.

U.S. Environmental Protection Agency. 1993b. Reregistration Eligibility Decision (RED) Paraquat Dichloride. List A Case 0262. Washington D.C., USA.

U.S. Environmental Protection Agency. 2003a. Interim Reregistration Eligibility Decision for Atrazine. EPA Case No. 0062. Washington D.C., USA.

U.S. Environmental Protection Agency. 2003b. White Paper on Potential Developmental Effects of Atrazine on Amphibians. Washington D.C., USA.

U.S. Environmental Protection Agency. 2009. National Primary Drinking Water Regulations. EPA 816-F-09-004. Washington D.C., USA.

University of South Florida. 2021. Skeleton of Frog. Available from: https://www.pinterest.com/pin/409616528582653178/ [4 February 2021]

van der Schalie, W. H., Gardner Jr, H. S., Bantle, J. A., de Rosa, C. T., Finch, R. A., Reif, J. S., Reuter, R. H., Backer, L. C., Burger, J., and Folmar, L. C. 1999. Animals as sentinels of human health hazards of environmental chemicals. Environmental Health Perspectives, 107(4): 309–315.

van Dijk, P. P., Iskandar, D., Inger, R. F., Lau, M. W. N., Ermi, Z., Baorong, G., Dutta, S., Manamendra-Arachchi, K., de Silva, A., Bordoloi, S., Kaneko, Y., Matsui, M., and Khan, M. S. 2004. *Fejervarya limnocharis*. The IUCN Red List of Threatened Species, 2004: e.T58275A86154107.

Venturino, A., Enrique, R., de Castro, A. C., Anguiano, O. L., Gauna, L., de Schroeder, T. F., and de D'Angelo, A. M. P. 2003. Biomarkers of effect in toads and frogs. Biomarkers, 8(3–4): 167–186.

Vismara, C., Battista, V., Vailati, G., and Bacchetta, R. 2000. Paraquat induced embryotoxicity on *Xenopus laevis* development. Aquatic Toxicology, 49(3): 171–179.

Walter, H., Harnickell, E., and Mueller-Dombois, D. 1975. Climate-diagram Maps of the Individual Continents and the Ecological Climatic Regions of the Earth 1st ed. vol. 8. Verlag, Germany: Springer, pp. 37.

Wang, Y. S., Jaw, C. G., and Chen, Y. L. 1994. Accumulation of 2, 4-D and glyphosate in fish and water hyacinth. Water, Air, and Soil Pollution, 74(3): 397–403.

Watts, M. 2011. Paraquat. Penang, Malaysia: Pesticide Action Network Asia and The Pacific, pp. 43.

Wehtje, G., Leavitt, J., Spalding, R., Mielke, L., and Schepers, J. 1981. Atrazine contamination of groundwater in the Platte Valley of Nebraska from non-point sources. Studies in Environmental Science, 17(1981): 141–145.

Wipatayotin, A. (4 March 2018). Group Urges Ban on Pair of Pesticides. Bangkok Post. Available from: https://www.bangkokpost.com/thailand/general/1421903/group-urges-ban-on-pair-of-pesticides

Won, S. J., Novillo, A., Custodia, N., Rie, M. T., Fitzgerald, K., Osada, M., and Callard, I. P. 2005. The freshwater mussel (*Elliptio complanata*) as a sentinel species: Vitellogenin and steroid receptors. Integrative and Comparative Biology, 45(1): 72–80.

Worldometers. 2018. South-Eastern Asia Population [Online]. Available from: https://www.worldometers.info/world-population/south-eastern-asia-population/ [29 September 2021].

Wu, J. P., Luo, X. J., Zhang, Y., Chen, S. J., Mai, B. X., Guan, Y. T., and Yang, Z. Y. 2009. Residues of polybrominated diphenyl ethers in frogs (*Rana limnocharis*) from a contaminated site, South China: Tissue distribution, biomagnification, and maternal transfer. Environmental Science & Technology, 43(14): 5212–5217.

Zhelev, Z., Popgeorgiev, G., Arnaudov, A., Georgieva, K., and Mehterov, N. 2015a. Fluctuating asymmetry in *Pelophylax ridibundus* (Amphibia: Ranidae) as a response to anthropogenic pollution in South Bulgaria. Archives of Biological Sciences, 67(3): 1009–1023.

Zhelev, Z., Popgeorgiev, G., and Mehterov, N. 2015b. Changes in the hepatosomatic index and condition factor in the populations of *Pelophylax ridibundus* (Amphibia: Ranidae) from anthropogenically polluted biotopes in Southern Bulgaria. Part II. Bulgarian Journal of Agricultural Science, 21(3): 534–539.

Zhelev, Z., Tsonev, S., and Angelov, M. 2019. Fluctuating asymmetry in *Pelophylax ridibundus* meristic morphological traits and their importance in assessing environmental health. Ecological Indicators, 107(105589): 1–14.

Zhelev, Z., Tsonev, S., and Arnaudova, D. 2017. Health status of *Pelophylax ridibundus* (Pallas, 1771) (Amphibia: Ranidae) in a rice paddy ecosystem in Southern Bulgaria: Body condition factor and fluctuating asymmetry. Acta Zoologica Bulgarica, 8: 169–176.

VITA

NAME	Luhur Septiadi
DATE OF BIRTH	15 September 1997
PLACE OF BIRTH	Malang, Indonesia
INSTITUTIONS ATTENDED	B.Sc. (Biology), Department of Biology, Faculty of Science and Technology, Maulana Malik Ibrahim State Islamic University of Malang, Indonesia, 2019
HOME ADDRESS	Jl. Melati No.57 Kebonsari RT03/RW01 Tumpang district, Malang Regency, East Java Province, Indonesia, 65156
PUBLICATION	PROCEEDINGS: Septiadi, L., Thammachoti, P., Claude, J., & Kitana, N. (2021). Health status of the rice frog Fejervarya limnocharis in Nan Province, Thailand, during peak herbicide utilization period. Proceedings, the 47th International Congress on Science, Technology, and Technology-based Innovation (STT47) - Sciences for SDGs: Challenges and Solutions, Nakhon Pathom, Thailand. pp. 364–373. ABSTRACTS: Septiadi, L., Thammachoti, P., & Kitana, N. (2021). Herbicides and population health of frog, Fejervarya limnocharis, in paddy fields at Northern Thailand. Abstract, the 11th Congress of Toxicology in Developing Countries (CTDC11), June 13–16, 2021, Kuala Lumpur, Malaysia. [Oral Presenter] Septiadi, L., Thammachoti, P., Claude, J., & Kitana, N. (2021). Health status of the rice frog Fejervarya limnocharis in Nan Province, Thailand, during peak herbicide utilization period. Abstract, the 47th International Congress on

Science, Technology, and Technology-based Innovation (STT47) - Sciences for SDGs: Challenges and Solutions, October 5–7, 2021, Kasetsart University, Nakhon Pathom, Thailand. [Oral Presenter]

AWARD RECEIVED

2018 & 2021: Equipment Grant Awardee of IDEAWILD Biodiversity Conservation Organization, Fort Collins, California, USA

2019: Best Graduate of Department of Biology, Maulana Malik Ibrahim Malang State Islamic University Malang, Indonesia

2019-2021: Awardee of Chulalongkorn University's Graduate Scholarship Program for ASEAN and Non-ASEAN Countries from Chulalongkorn University, Thailand

2021: Awardee of the 90th Anniversary of Chulalongkorn University Scholarship from the Graduate School, Chulalongkorn University, Thailand