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Altered Immune Response of the Rice Frog *Fejervarya limnocharis* Living in Agricultural Area with Intensive Herbicide Utilization at Nan Province, Thailand

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Abstract

Herbicides (atrazine, glyphosate and paraquat) have been intensively used in Nan Province for a long time. Prior observations indicated that herbicide contamination and adverse health effects were found on the rice frog *Fejervarya limnocharis* living in paddy fields at Nan Province. Contamination of herbicides may influence disease emergence by acting directly or indirectly upon the immune system of amphibian or by causing disruptions in homeostasis, it is thus interesting to investigate potential effects of herbicide contamination in Nan Province on immune responses of the rice frog living in agricultural areas. Frogs were caught from a paddy field with no history of herbicide utilization (reference site) and a paddy field with intensive herbicide utilization (contaminated site) during 2010-2011. After dissection, frog livers were fixed in 10% neutral buffer formalin, processed by paraffin method and stained with hematoxylin and eosin. Number of melanomacrophage and melanomacrophage center (MMC) were counted under a light microscope and used as markers of non-specific immune response. It was found that there was no significant sex-related difference in these numbers. However, there were significant seasonal differences in these numbers in both reference and contaminated site frogs, suggesting that seasonal difference in herbicide usage tend to affect frog's immune system in agricultural areas. Furthermore, numbers of melanomacrophage and MMC in early wet, late wet and early dry periods were markedly higher in the contaminated site frogs compared to those of the reference site frogs. The observation on amphibian's immune response to environmental contaminants could indicate the impacts of herbicide utilization on other vertebrates, as well as its role in amphibian declines.

Keywords: agrochemicals; amphibian; liver; melanomacrophage; sentinel species

1. Introduction

At present, agrochemicals (herbicide, insecticide and fungicide) have been intensively used in many agricultural areas of Thailand, especially Nan Province. Previous report on agrochemicals import to Nan Province showed that 92.04% of agrochemicals is herbicides, especially atrazine, paraquat and glyphosate (Chanphong, 2008). Agrochemicals usage may lead to accumulation in environment and non-target organism, including human. Amphibians are good indicators of pollution since their skin is highly permeable and they have life cycle in both aquatic and terrestrial habitats. Therefore, there are several routes that xenobiotics can enter their body (Rollins-Smith *et al.*, 2007). Amphibian's response to environmental contaminants could indicate the impacts on other vertebrates and human (Roy, 2002).

Chemical contaminants is one of factors involved in amphibian declines (Young *et al.*, 2001). Increases in susceptibility to pathogens may resulted from stressful habitat conditions and subsequent disruption of immune response (Falso *et al.*, 2011). Environmental contaminants could suppress immune defenses and magnify the effects of disease in several animals including amphibian. Therefore, immune system of amphibian may be a sensitive target of poisoning by environmental contaminants (Rollins-Smith *et al.*, 2007). However, there are still only a few reports on amphibian immune response to environmental contamination. Examples of these works included effects of pesticides on *Rana pipiens* (Gilbertson *et al.*, 2003; Christin *et al.*, 2004) and *Xenopus laevis* (Christin *et al.*, 2004), effects of agricultural activities on *R. catesbeiana* (Falso *et al.*, 2011), and effects of environmental cadmium contamination on

F. limnocharis (Othman, 2009). Some of these works showed the link between contamination and change in number of melanomacrophage center (MMC), or an accumulation of pigmented macrophages that posses different types of granules inside cytoplasm including melanin, hemosiderin and lipofuscin. MMC is usually found in poikilothermic vertebrates such as fish, amphibian and reptile (Wolke, 1992; Agius and Roberts, 2003). MMC's function has been linked with immunity, cell and compound storage, destruction, and detoxification and iron recycling (Agius and Roberts, 2003). Increase in number of MMC could be associated with environmental stressors. The use of MMC in environmental monitoring program is thus recommended.

The rice frog, *F. limnocharis*, a common amphibian species of South and Southeast Asia has been regarded as a good sentinel species for environmental contamination (Othman, 2009; Thammachoti, 2012). According to the National Research Council (1991), justification of using the rice frog as sentinel species included a measurable response to the toxicants, a territory or home range that overlaps the area to be monitored, easiness to enumerate and capture and sufficient population size and density to permit enumeration.

At Nan Province, there are previous reports on herbicide contamination in both environment (atrazine) and the rice frog tissue (atrazine, glyphosate and paraquat). This environmental stress (herbicides) could affect frog health as revealed from a decrease in condition factor and an increase in hepatosomatic index (Thammachoti *et al*., 2012). Therefore, it is interesting to determine the influence of herbicide utilization on immune system of the rice frogs living in agricultural areas at Nan province. This study thus aimed to compare non-specific immune response (numbers of melanomacrophage and MMC) of the rice frogs, *F. limnocharis* living in agricultural areas with different degree of herbicide utilization at Nan Province.

2. Materials and Methods

2.1. Study sites

The study sites located in Wiang Sa District, Nan Province included 1) a contaminated site which is an agricultural area with intensive herbicide usage located in San Subdistrict (UTM 0687729 2054283, zone line 47Q), and 2) a reference site which is an organic agricultural area with no history of herbicide usage for almost 10 years located in Lainan Subdistrict (UTM 0686779 2047187, zone line 47Q) (Fig. 1). Prior study indicated that atrazine residue (0.15 mg/L) was found in surface water at the contaminated site during late dry season while no herbicide residue was found in the reference site in any season (Maneein *et al*., 2011; Thammachoti, 2012). Further analyses showed that frog from the contaminated site tended to have higher level glyphosate and markedly higher level of atrazine and paraquat in the tissue compared to those in the reference site (Thammachoti, 2012).

2.2. Rice frog collection

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

2.3. The number of melanomacrophage and melanomacrophage center (MMC) in liver of the rice frogs

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

2.4. Statistical analysis

Data were tested for normal distribution and homogeneity of variance. Mean comparison between

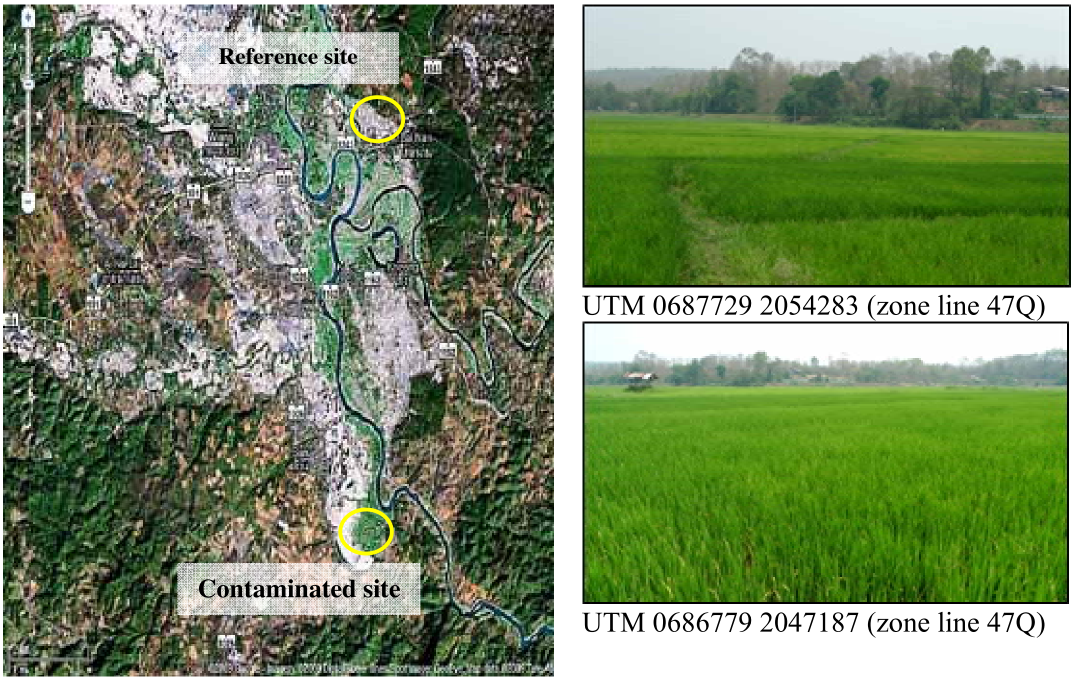

Figure 1. Study sites included a reference site which is a paddy field in Lainan Subdistrict with no history of herbicide usage and a contaminated site in San Subdistrict with intensive herbicide usage.

sex was determine using Student's *t*-test. Mean comparison among sites and seasons was determined using two-way ANOVA followed by Student-Newman-Keuls Method. Correlation of melanomacrophage and MMC numbers vs. herbicide residues (Thammachoti, 2012) was determined using Pearson's correlation.

3. Results and Discussion

Melanomacrophage and MMCs are focal accumulations of pigmented macrophages in liver, spleen and kidney of fish (Agius and Roberts, 2003) and in liver of frog (Loumbourdis and Vogiatzis, 2002). These pigment cells belong to the exocutaneous pigmentary system of the lower vertebrates (Breathnach, 1988). In this study, it was found that melanomacrophages and MMCs were found in liver of the rice frog from both reference and contaminated sites (Fig. 2a and 2b). To determine if sex had a significant effect on this parameter, comparison for number of melanomacrophages and MMCs was made and shown that no significant sex-related difference in these numbers was found (Student's *t*-test, $p>0.05$, data not shown). However, there were significant site-related differences in these numbers, with the higher numbers of melanomacrophages and MMCs in the contaminated site frog ($p<0.05$, power of test [$\alpha=0.05$] = 0.89), except in late dry period (January 2011). Furthermore, there were significant seasonal differences in these numbers in both reference and contaminated site frogs (Table 1),

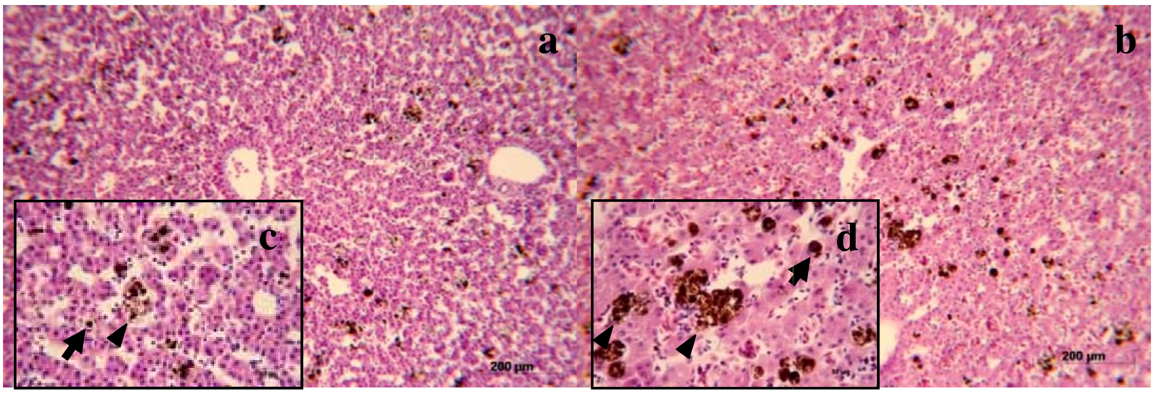

Figure 2. Micrographs of liver (H&E staining) of the rice frog, *F. limnocharis*, caught from the reference site (a = 100X, c = 400X) and the contaminated site (b = 100X, d = 400X) in Nan Province, Thailand. Melanomacrophages (arrow) and melanomacrophage centers (MMCs; arrowhead) are shown.

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

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

Remarks:

Significant seasonal difference within the same site (two-way ANOVA, $p<0.05$, power of test [$\alpha=0.05$] = 0.89) is indicated by different superscript letters. (Significant seasonal-related difference within contaminated site is indicated by the capital letter and significant seasonal-related difference within reference site is indicated by the small letter). Significant site-related difference within the same period (two-way ANOVA, $p<0.05$) is indicated by an asterisk (*).

which may relate to the difference in agricultural activities of both study sites (data not shown).

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

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

In this study, influence of herbicide utilization on immune system of the rice frogs was examined. The results showed that there were significant seasonal and site-related differences in numbers of melanomacrophage and MMC in liver of the rice frogs. This suggests

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

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

that seasonal and site-related difference in herbicide utilization tend to affect frog's immune system in agricultural areas. For seasonal difference, it was well known that liver of the frog is a very plastic organ in which both the epithelial and the histiocytic components are very sensitive to certain annual biological rhythms (i.e. reproduction). Liver melanomacrophages of *Rana esculenta* tended to show difference in metabolically and cytokinetically active cell population during the annual cycle. This phenomenon is probably regulated by highly integrated mechanisms responsible for maintaining a functional homeostatic balance during the different adaptation responses (Barni *et al*., 2002). In addition, MMC's function has been linked with cell and compound storage, destruction, and detoxification and iron recycling (Agius and Roberts, 2003). MMCs of teleost fish were reported to trap and retain antigens during the immune response and were closely associated with immunoglobulin-secreting cells (Vigliano *et al*., 2006). Since MMCs could play roles in immunity (inflammatory and humoral responses; Agius and Roberts, 2003), an increase in number and area of MMCs could be sensitive to environmental stressors.

Unfortunately, there were only a few studies in regard to the use of numbers of MMCs as immunological biomarkers the in frogs and other amphibians (Barni *et al*., 2002; Loumbourdis and Vogiatzis, 2002; Grassi *et al*., 2007). Nevertheless, pigmented macrophage accumulations were used as potential marker of fish health (Blazer and Dethloff, 2000). Studies about MMCs as biomarkers in many fish species demonstrated that the occurrence of MMCs may vary depending on many factors namely the nutritional status, health or size of a particular fish species (Agius 1979; Agius 1980; Agius and Roberts 1981; Wolke *et al*., 1985). The poor health fish or fish with nutritional deficiencies and larger fish tend to have more or larger MMCs. Moreover, the number and/or size of MMCs in many fish increase with age (Brown and George, 1985; Blazer *et al*., 1987). Importantly, numerous studies had documented an increase in their number, size or hemosiderin content in fish collected at contaminated sites compared to those collected at reference sites. MMCs were used as a potentially sensitive biomarkers of contaminant exposure and a potential immunotoxic biomarkers (Blazer *et al*., 1997; Matavulj *et al*., 2005). Since there were only a few extensive, controlled attempts to produce MMCs or to study their kinetics by chronic exposure to contaminants known to exist in polluted environments, the use of MMCs as biomarkers of vertebrate health and environmental degradation should be further examined using the systematic controlled experiment (Blazer and Dethloff, 2000).

Prior reports showed that herbicide contaminations were found in environment and tissue of the rice frog, *F. limnocharis*, living in paddy fields at Nan Province. Negative impacts on the frog's health were evidenced (Thammachoti *et al*., 2012). The current findings of significant seasonal and site-related differences in numbers of melanomacrophages and MMCs suggested potential effect of herbicide on non-specific immune response of the frog. Further study on influence of herbicide contamination on immune system of the rice frog should be performed with other techniques to examine both non-specific and specific immune response of the rice frog.

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