ผลของอะทราซีนต่อการเจริญระยะต้นและการเจริญของอวัยวะสร้างเซลล์สืบพันธุ์ของกบนา *Hoplobatrachus rugulosus* (Wiegmann, 1834)

นายกฤษฎา คทาวุธพูนพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF ATRAZINE ON THE EARLY DEVELOPMENT AND GONAD DEVELOPMENT OF RICE FIELD FROG *Hoplobatrachus rugulosus* (Wiegmann, 1834)

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
Department of Biology
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Thesis Title	**EFFECTS OF ATRAZINE ON THE EARLY DEVELOPMENT AND GONAD DEVELOPMENT OF RICE FIELD FROG *Hoplobatrachus rugulosus* (Wiegmann, 1834)**
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Field of Study	Zoology
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กฤษฎา คทาวุธพูนพันธ์ : ผลของอะทราซีนต่อการเจริญระยะต้นและการเจริญของอวัยวะสร้างเซลล์สืบพันธุ์ของกบนา *Hoplobatrachus rugulosus* (Wiegmann, 1834). (EFFECTS OF ATRAZINE ON THE EARLY DEVELOPMENT AND GONAD DEVELOPMENT OF RICE FIELD FROG *Hoplobatrachus rugulosus* (Wiegmann, 1834)) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. อรวรรณ สัตยาลัย, 120 หน้า.

ปัจจุบันประชากรและความหลากหลายของสัตว์สะเทินน้ำสะเทินบกมีแนวโน้มลดลงทั่วโลก การปนเปื้อนของสารเคมีในสิ่งแวดล้อมเป็นปัจจัยหนึ่งที่มีผลต่อการลดลงของสัตว์ในกลุ่มดังกล่าว อะทราซีน (atrazine) เป็นสารเคมีสังเคราะห์ในกลุ่มไทรอะซีนซึ่งใช้เป็นสารกำจัดวัชพืชอย่างแพร่หลายทั้งในประเทศไทยและหลายประเทศทั่วโลกการใช้อะทราซีนพบมากในช่วงก่อนฤดูฝนเล็กน้อยจึงทำให้พบการปนเปื้อนอะทราซีนในแหล่งน้ำธรรมชาติปริมาณสูงเนื่องจากการชะล้างของน้ำฝนลงสู่แหล่งน้ำ ซึ่งฤดูดังกล่าวเป็นฤดูผสมพันธุ์ของสัตว์สะเทินน้ำสะเทินบก ดังนั้นสัตว์ในกลุ่มนี้จึงมีโอกาสสูงที่จะได้รับผลจากอะทราซีน การศึกษาครั้งนี้เลือกใช้กบนา *Hoplobatrachus rugulosus* (Wiegmann, 1834) เป็นสัตว์ทดลองเพื่อศึกษาผลของอะทราซีน การศึกษาแบ่งเป็นสามส่วน ได้แก่ การศึกษาการเจริญระยะต้นในห้องปฏิบัติการเพื่อหาช่วงเวลาที่ใช้การเจริญระยะต่างๆ เพื่อนำข้อมูลไปใช้ในการทดลองต่อไป การทดลองที่สองศึกษาผลของอะทราซีนต่อการเจริญระยะต้นของกบนาโดยวิธี FETAX โดยนำไข่กบนามาทดสอบด้วยอะทราซีนที่ความเข้มข้นเท่ากับที่มีรายงานพบในธรรมชาติได้แก่ 0.001, 0.01, 0.1, 10, 100 และ 1,000 ppb เป็นเวลา 96 ชั่วโมง ผลการศึกษาพบว่าอะทราซีนทุกความเข้มข้นที่ใช้ในการทดลองนี้ไม่ได้ทำให้อัตราการตาย ความยาวจากจมูกถึงช่องเปิดทวารหนัก และความผิดปกติของการเจริญที่พบในกลุ่มทดลอง มีความแตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ถึงแม้ว่าจะพบความผิดปกติได้แก่ลักษณะแกนหางโค้งงอและลักษณะท้องบวมเฉพาะในกลุ่มทดลองทุกความเข้มข้นยกเว้นที่ 0.01 ppb การทดลองที่สามศึกษาผลของอะทราซีนต่อการเจริญของอวัยวะสร้างเซลล์สืบพันธุ์และการเจริญในช่วงตัวอ่อนจนกระทั่งมีเมทามอร์โฟซิสเป็นตัวสำเร็จโดยใช้ความเข้มข้นของอะทราซีนที่ 0.001, 0.01, 0.1, 10, 25, 100 และ 1,000 ppb ผลการศึกษาพบว่าอะทราซีนไม่ทำให้เกิดความผิดปกติในการเจริญของอวัยวะสร้างเซลล์สืบพันธุ์ และไม่ทำให้กลุ่มทดลองมีการเจริญจนเป็นตัวสำเร็จแตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญ($p<0.05$) ผลการศึกษาที่ได้สรุปได้ว่าอะทราซีนบริสุทธิ์ในความเข้มข้นที่พบในธรรมชาติที่ใช้ในการทดลองครั้งนี้ไม่ทำให้เกิดการเจริญที่ผิดปกติต่อเอ็มบริโอ ตัวอ่อน และอวัยวะสร้างเซลล์สืบพันธุ์ของกบนาที่เลี้ยงในห้องปฏิบัติการ

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต กฤษฎา คทาวุธพูนพันธ์

สาขาวิชา สัตววิทยา ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก อรวรรณ ส.

ปีการศึกษา 2551

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KEYWORDS : ATRAZINE/ *Hoplobatrachus rugulosus* /DEVELOPMENT
KRITSADA KATAWUTPOONPHAN : EFFECTS OF ATRAZINE ON THE EARLY DEVELOPMENT AND GONAD DEVELOPMENT OF RICE FIELD FROG *Hoplobatrachus rugulosus* (Wiegmann, 1834).
ADVISOR : ASST. PROF. ORAWAN SATAYALAI, Ph.D., , 120 pp.

Amphibians have declined dramatically in many areas around the world. Agrochemicals and chemicals contamination to the environment are also considered as a disastrous causes of amphibian declines. Atrazine, a triazine-herbicide is the most commonly used herbicide in the world and probably in Thailand. Atrazine is generally applied in rainy season when amphibians are congregating and preparing for the breeding. Therefore, frogs either in the agricultural fields or the adjacent wetlands could be exposed to atrazine. This study designed to determine the effects of atrazine on the embryonic and larval development in the rice field frog *Hoplobatrachus rugulosus* (Wiegmann, 1834), a common frog species in Thailand. The study was divided into 3 parts. The first part was to determine the normal embryonic and larval developmental of *H. rugulosus* to find out each time of developmental stage for the following experiments. The second part was to determine the effects of atrazine on early development of *H. rugulosus*. The early embryos at mid-blastula were treated with the ecologically relevant nominal concentrations *i.e.* 0.001, 0.01, 0.1, 10, 100, and 1,000 ppb using FETAX protocol for 96 hours. The result shown that the nominal concentrations of atrazine used in this experiment did not cause significant statistical effects ($p<0.05$) such as mortality rate, snout-vent length and developmental abnormalities, even though certain abnormalities i.e. tail flexure and abdominal edema were found in all treatment groups (except 0.01 ppb). The last part was to determine the effects of atrazine on gonad development and later developmental stages. The early embryos were treated with the nominal concentrations of atrazine *i.e.* 0.001, 0.01, 0.1, 10, 25, 100, and 1,000 ppb, respectively. The embryos were treated with atrazine until the animal completed metamorphosis. The results showed that the nominal concentrations of atrazine did not cause any effects to the metamorphosis and gonad development of *H. rugulosus* ($p<0.05$). In conclusion, it is suggested that pure atrazine at the ecologically relevant concentrations were not capable of causing direct effects to *H. rugulosus* development in laboratory condition.

Department : Biology Student's Signature Kritsada Katawutpoonpha
Field of Study : Zoology Advisor's Signature Orawan Satayalai
Academic Year : 2008

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List of Abbreviations

µg	microgram
2-D	2, 4-Dichlorophenoxyacetic acid
atz	atrazine
cm	centimeter
conc	concentration
d	day
DNA	deoxyribonucleic acid
EC_{50}/ED_{50}	half maximal effective concentration/ dose
hr	hectare
hr	hour
IUCN	International Union for Conservation of Nature
km	kilometer
L/l	liter
LC_{50}/LD_{50}	median lethal concentration/ median lethal dose
LH	luteinizing hormone
LOAEC	lowest observable adverse effect concentration
m	meter
mg	milligram
min	minute
ml	milliliter
mm	millimeter
ng	nanogram
NOAEC	no observed adverse effect concentration
ppb	part per billion (equal to µg/l)
ppm	part per million (equal to mg/l)
ppt	part per trillion (equal to ng/l)
temp	temperature
U.S. EPA	United States Environmental Protection Agency

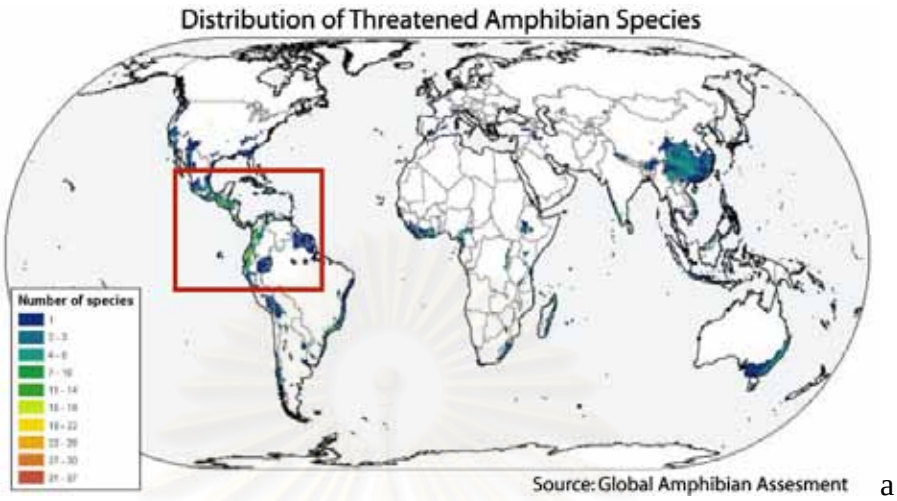
CHAPTER I

GENERAL INTRODUCTION

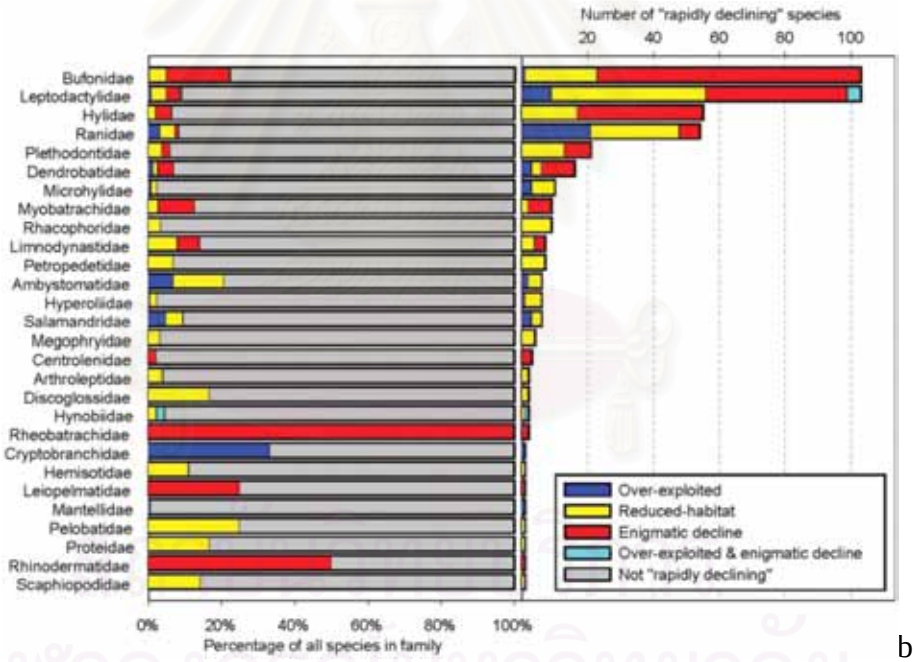
A. Global Decline of Amphibians

Amphibians are a group of vertebrates that includes frogs, toads, salamanders, newts and caecilians. They play an important role as both prey and predator. Moreover, amphibians provide many ecological services such as the biological control of insects (Young *et al.*, 2004). The estimated number of all known global amphibian species is approximatly 6,300 species (Stuart *et al*., 2009). However, in recent years the phenomenon of amphibian decline whether at a species or a population level has been recognized (Fig 1-1) and this crisis has become a major concern for many biologists (Hayes *et al*., 2002a; Baillie *et al*., 2004; Stuart *et al*., 2004; Beebee and Griffiths, 2005). At least 1,856 species which is 32% of all species are reported to be threatened (Baillie *et al.*, 2004). Furthermore, the IUCN Global Amphibian Assessment indicated that 43 percent of all amphibian species are in population decline, and at least nine species have gone extinct since 1980 and 113 species have not been found in recent years suggesting that they too may have already become extinct (Baillie *et al.*, 2004).

There are many possible causes of amphibian declines for example; introduced species, diseases, climate change and habitat destruction (Beebee and Griffiths, 2005). Furthermore, other major causes of amphibian decline are chemical and agrochemical pollutants which contaminate the natural amphibian habitat (Beebee and Griffiths, 2005). Due to the growing economic demand for agro-industrial goods, large amounts of agrochemicals such as fertilizers, insecticides, and herbicides have been applied on



a



b

Figure 1-1: (a) World global amphibian decline map,

(b) Percentages and numbers of rapidly declining species in amphibian families (with at least one rapidly declining species), broken into groups reflecting the dominant cause of rapid decline: overexploitation, habitat loss, or enigmatic decline (Stuart *et al.*, 2004).

farmland. As a result, amphibians that live adjacent to such farmlands suffer from the agrochemicals used. For example, Johnson *et al.* (2007) reported that heavy fertilizer application caused high nutrient accumulation in farmland and this condition in turn can promote the infection of trematode parasites in frog tadpoles. Moreover, this condition has interfered with limb development processes and resulted in malformed adults with extra or missing limbs.

Atrazine is one of the most heavily used herbicides in the U.S. and probably in the world (Hayes *et al.*, 2002a; Dorsey *et al.*, 2003; Steeger and Teitge, 2003; Lenkowki *et al*., 2008, Oka *et al*., 2008). Atrazine has been used for over 40 years and currently it is used in more than 80 countries (Hayes, 2002a). With these heavy applications, atrazine has commonly been detected in the surface waters of agricultural watersheds. In Thailand, atrazine has been also heavily used for agricultural purpose according to the data from the Department of Agriculture (2007) more than three million kilograms of chemically effective atrazine was imported (Table 1-1). However, few data are available regarding the effect of atrazine on organisms in Thailand.

Table 1-1: Import data of atrazine from the Customs Department of Thailand. (Department of Agriculture Thailand, 1998; 1999; 2000; 2001; 2002; 2003; 2004; 2005; 2006; 2007)

year	Quantity (kg)	Active compound quantity (kg)
1998	803,680	642,944.00
1999	1,030,320	824,256.00
2000	1,568,020	1,199,856.00
2001	2,129,695	1,832,225.50
2002	1,688,500	1,425,750.00
2003	2,364,450	1,998,320.00
2004	1,997,800	1,670,460.00
2005	2,398,300	2,019,410.00
2006	2,839,161	2,392,307.00
2007	3,686,650	3,049,135.00

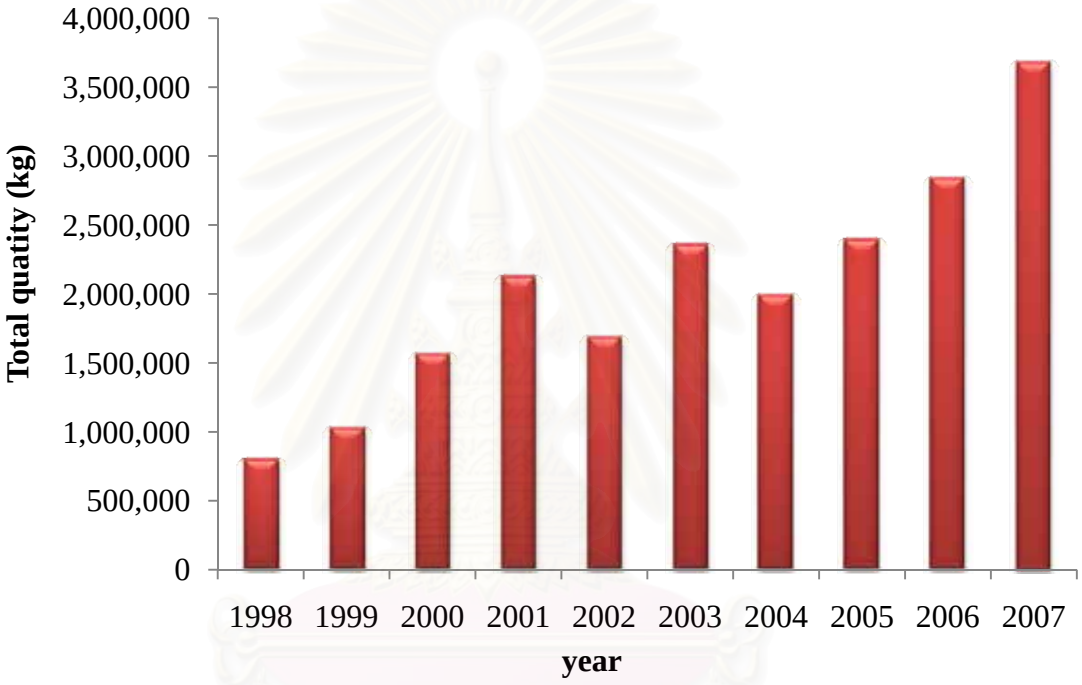
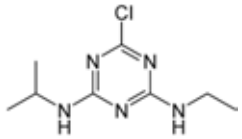


Figure 1-2: Import data of atrazine from the Customs Department of Thailand from 1998 to 2007

B. Atrazine information

Formula: 

Chemical family: Triazines

Empirical formula: $C_8H_{14}ClN_5$

Molecular weight: 215.7

IUPAC name: 1-chloro-3-ethylamino-5-isopropylamino-2, 4, 6-triazine

Trade and other names: include Aatrex, Aktikon, Alazine, Atred, Atranex, Atrataf, Atratol, Azinotox, Crisazina, Farmco Atrazine, G-30027, Gesaprim, Giffex 4L, Malermais, Primatol, Simazat, and Zeapos.

Physical Properties

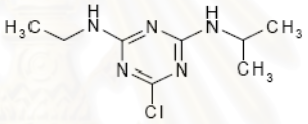
Atrazine is a white crystalline solid. Atrazine is produced commercially in the United States as a technical-grade chemical with a purity of 92–97% to 99.9% active ingredients (Dorsey *et. al.*, 2003). Impurities in the former formulation included dichlorotriazines, hydroxytriazines, and tris (alkyl) aminotriazines. Information regarding the physical and chemical properties of atrazine is located in Table 1-2.

Atrazine is a selective, pre-and post-emergence herbicide used on a variety of terrestrial food crops, non-food crops, forests, residential lawns, golf courses, recreational areas, rights-of-way and rangeland. It is available in various forms as a dry flowable powder, a flowable liquid, and water dispersible granular and wettable powder formulations (Dorsey *et. al.*, 2003). Atrazine's primary mode of action in plants is through inhibition of photosynthesis by disruption of the Photosystem II

pathway (Steeger and Tietge, 2003). Atrazine is characterized as a persistent, mobile compound that may be transported to surface water via runoff, spray drift and atmospheric deposition. Because of the chemical's resistance to abiotic routes of degradation it has been widely detected in surface waters throughout the aquatic environment underlining its associated exposure potential for aquatic organisms.

Table 1-2: Chemical and physical information of atrazine

(modified from Dorsey *et al*., 2003.)

Characteristic/ Properties	Information
Chemical name	Atrazine
Synonyms	6-Chloro-n-ethyl-n'-(1-methylethyl)-triazine-2,4-diamine; 2-Chloro-4-ethylamino-6-isopropylamine-s-triazine; 2-Chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine; 2-Chloro-4-(ethylamino)-6-(isopropylamino)-triazine; Chloro-4-(propylamino)-6-ethylamino-s-triazine; Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine; Butyl-n-(acetyl)-aminopropionic acid
Registered trade names	Aatrex®, Aatram®, Atratol®, Gesaprim®
Chemical structure	
Identification numbers	
CAS registry	1912-24-9
NIOSH RTECS	XY5600000
Molecular weight	215.69
Colour	White colorless
Physical state	Colorless powder, Colorless crystals
Melting point	173–175 °C
Density	1.23 g/cm3 (22 °C)
Odor	Odorless
Solubility	
at 22 °C	Soluble in water (34.7 mg/l)
at 25 °C	Soluble in ethylacetate (24 g/l),acetone (31 g/l), dichloromethane (28 g/l), ethanol (15 g/l), toluene (4 g/l), n-hexane (0.11 g/l), and n-octanol (8.7 g/l)
at 27 °C	Soluble in n-pentane (360 mg/l), diethyl ether (12,000 mg/l), methanol (18,000 mg/l), ethyl acetate (28,000 mg/l), chloroform (52,000 mg/l), and dimethyl sulfoxide (183,000 mg/l)
Vapor pressure at 25 °C	2.89×10^{-7} mmHg
Henry's Law constant at 25 °C	2.96×10^{-9} atm-m3/mol
pKa	1.68
Hydrolysis rate constant at 25 °C	2.735x10-11 cm^3/molecule-second (estimated)

C. Environmental fate and residue of atrazine.

After atrazine is applied in agricultural areas, appreciable quantities could reach the ecosystem through air transport or also be leached from the soil by rainfall and enter aquatic bodies such as rivers, streams, lakes, etc. Moreover, atrazine may be transferred from the upper to the lower soil levels and enter the groundwater (Dorsey *et al.*, 2003). In addition, it can be volatized from agricultural soils to the atmosphere (Glotfelty *et al.*, 1989; Weinhold and Gish, 1994).

Atrazine has been detected in the atmosphere both near to and at a considerable distance from applied areas, up to 100–300 km (Thurman and Cromwell, 2000) as a result of atmospheric transport. This atmospheric transported atrazine was found during the first month after application. However, in some cases it was found 4 to 8 months later (Dorsey *et al.*, 2003). Atrazine can exist in both particulate and vapor phases in the atmosphere. Up to 14% of the applied atrazine can be volatized from conventionally tilled fields, but only 9% of the total was volatilized from no-till fields (Weinhold and Gish, 1994). It could be assumed that if only 2.4% of atrazine is volatized, then taking the total atrazine used in the U.S. in 1997 this would result in more than 700 tons of atrazine being dispersed in the atmosphere (Dorsey *et al.*, 2003). Atrazine residues in the atmosphere were observed by Trevisan *et al.* (1993) who demonstrated that 1 out of 10 rainwater samples of 146 samples collected contained atrazine with concentrations ranging from 0.15 to 1.99 ppb, with a median concentration of 0.88 ppb. These amounts fluctuated with the season with the highest concentrations in June, following the earlier spring-time application to the crop. Trochimowicz *et al.* (2001) reported that atrazine concentrations in the air in regions

in and around Paris, France ranged from just above the detection limit of approximately 0.03 ng/m^3 to more typical concentrations of 0.20–0.32 ng/m^3.

Atrazine can remain in the soil with a half life of 4-57 weeks, however under certain parameter even longer than 1 year under cold weather conditions (Dorsey *et al.*, 2003). Atrazine residues vary in soils, depending on usage and exposure to climatic patterns that may lead to atrazine deposition. Frank and Sirons (1985) reported concentrations in soils ranged from 0.83 μg/g, 6 days following application of 1.1 kg/ha, to 0.5 μg/g after 2 months, to 0.08 μg/g after 12 months and concentrations have been shown to slowly decline over a periods of 12 months. Atrazine is moderately to highly mobile in soil containing a low clay and organic matter content because atrazine does not adsorb to soil particles; consequently, increasing its high potential to enter ground water.

Atrazine can also reach aquatic systems through runoff and has been identified in both ground water and surface water. It is moderately soluble in water and it's degradation in surface water is slow. There have been reports that atrazine has a long residence time (Dorsey *et al.*, 2003). Atrazine concentrations in surface runoff were highest following applications in the fields (Gaynor *et al.*, 1995). Gaynor *et al.* (1995) reported the highest maximum amount of atrazine observed in surface runoff was 400 to 700 ppb depending on the agricultural practices used. They mention that in the receiving streams, atrazine concentrations were about 10-folds lower than the surface runoff concentrations. This difference was a result of soil sorption and other losses that occurred prior to the surface runoff reaching water surface bodies (Gaynor *et al.*, 1995), and not simply dilution into the larger quantity of receiving waters.

Concentrations of atrazine in surface waters fluctuate with the season depending on agricultural practices. The highest atrazine concentrations were observed in the weeks and months following application of the herbicide (Albanis *et al.*,1998; Battaglin and Goolsby, 1999). The detections of atrazine were observed prior to planting and shortly thereafter. For example, atrazine was detected in 91% of 55 surface water (river and stream) samples that were collected before crops were planted, and in 98% of 132 water samples collected within 2 weeks of crop planting. Following the growth season (at harvest), it was detected in only 76% of 145 of the water samples collected (Thurman *et al.*, 1991). The samples collected after the crops were planted contained an order of magnitude higher concentrations (median concentration approximately 4 µg/l). Frank *et al.* (1982) found 80% of agricultural watershed streams contained atrazine residues with the highest concentration at 33 ppb, and the average ranging from 1.1 to 1.6 ppb.

Atrazine has also been detected in oceans, at concentrations ranging from 1 to 100 ppt (ng/l) and in estuaries at concentrations of 200 ppt. Concentrations were generally higher closer to the shore (Bester and Huhnerfuss, 1993).

In Thailand, Poonsuk Haruetaithanasant (2005) reported atrazine residues in the Mae Klong and Songkram rivers in Thailand. The observation revealed that in the Mae Klong River in the rainy season, all samples were contaminated with atrazine with average concentration ranging from 0.0020 to 1.730 ppb. In the Songkram River, atrazine residues were from 0.0019 to 0.1326 ppb (Poonsuk Haruetaithanasant, 2005).

Table 1-3: Example of atrazine residues concentrations found in environment.

authors and year	source	concentration found (average or range)	month/ season	venue
Trevisan *et al.*, 1993	atmosphere ; rain water samples	0.15-1.99 ppb	Highest concentration in June	Italy
Trochomowitz *et al.*, 2001	atmosphere	0.03 ng/m^3 0.20-0.32 ng/m^3	N/A	Paris/ France
Frank and Sirons, 1985	soil	0.83 µg/g 6 months after applied 0.08 µg/g 12 months after applied		Canada
Frank *et al.*, 1982	water shed streams	1.1-1.6 ppb (highest 33 ppb)	N/A	Canada
Bester and Huhnerfuss, 1993	- estuaries - ocean	200 ppt 1-100 ppt	N/A	Germany
Thurman *et al.*, 1991	- water sample (after crop planted)	median 4 ppb	N/A	Mid-western, U.s.
Gaynor *et al.*, 1995	- surface water run off - recieving streams	400-700 ppb	N/A	Experimen-tal farm Canada
Poonsuk Haruethaithanasant, 2005	- river	0.0147 ppb	summer	Songkram river, Thailand
		No detection	rainy season	Songkram river, Thailand
		0.019 ppb	summer	Mae Klong river, Thailand
		0.239 ppb	rainy season	Mae Klong river, Thailand

D. Animal model: *Hoplobatrachus rugulosus* (Wiegmann, 1834)

General classification

Kingdom Animalia

Phylum Chordata

Class Amphibian

Order Anura

Family Dicroglossidae

Species: *Hoplobatrachus rugulosus* (Wiegmann, 1834)

Synonyms: *Rana chinensis* Osbeck, 1765; *Rana rugulosa* Wiegmann, 1834; *Rana tigrina* var. *pantherina* Steindachner, 1867; *Hydrostentor pantherinus* Steindachner, 1867; *Rana esculenta chinensis* Wolterstorff, 1906; *Rana burkilli* Annandale, 1910; *Rana tigerina* var. *burkilli* Boulenger, 1918; *Rana rugulosa* Annandale, 1918; *Rana (Rana) tigerina* var. *pantherina* Boulenger, 1920; *Rana tigrina rugulosa* Smith, 1930; *Rana tigerina rugulosa* Fang & Chang, 1931; *Rana tigerina pantherina* Taylor & Elbel, 1958; *Rana (Euphlyctis) rugulosa* Dubois, 1981; *Euphlyctis tigerina rugulosa* Poynton & Broadley, 1985; *Limnonectes (Hoplobatrachus) rugulosus* Dubois, 1987; *Tigrina rugulosa* Fei, Ye & Huang, 1990 *Hoplobatrachus rugulosus* Dubois, 1992*; Hoplobatrachus chinensis* Ohler, Swan & Daltry, 2002

Common Names**:** Rice field frog, Chinese edible Frog, East Asian bullfrog, Taiwanese Frog, I-san Field Frog, Rugosed Frog, Rugose Frog, Common lowland frog.

IUCN Red List Category & Criteria**:** Least Concern (Diesmos *et al.*, 2004)

This species is widespread from central, southern and south-western China including Taiwan, Hong Kong and Macau to Myanmar through Thailand, the Lao People's Democratic Republic, Vietnam and Cambodia and south to the Thai-Malay peninsula. It was introduced and established in Sabah, Borneo in the 1980's. It has also recently been introduced to the Philippines and to date has been reported in central Luzon and from Puerto Princesa City on Palawan. (Diesmos *et al.*, 2004) (Figure 1-2)**.**

It naturally lives in paddy fields, irrigation infrastructures, fishponds, ditches, floodplain wetlands, forest pools, and other wet areas. The tadpoles live in pools and ponds. The adults are effective predators on other species of frogs and its larvae prey on tadpoles of other species (Diesmos *et al.* 2004).

This frog is known as an edible frog. It is considered medium to large in size; with a weight range from 200-400 g, and a snout-vent length range from 80-100 mm (Hauy Hong Krai Royal Development Study Centre, 2002). The size of the adult male frog is smaller than the female. The colors are brownish olive on the back of head, somewhat lighter on the sides, with very slightly indefinite gray marks on the chin. The snout is oval with the nostrils nearer the eye than the snout tip, the tympanum is covered with skin. There are 4 and 5 digits respectively on arms and legs, with all digits rather pointed and without small discs. The arms and legs are moderately short

Figure 1-3: Geographic Range of *Hoplobatrachus rugulosus* (Wiegmann, 1834) (Diesmos *et al.*, 2004)

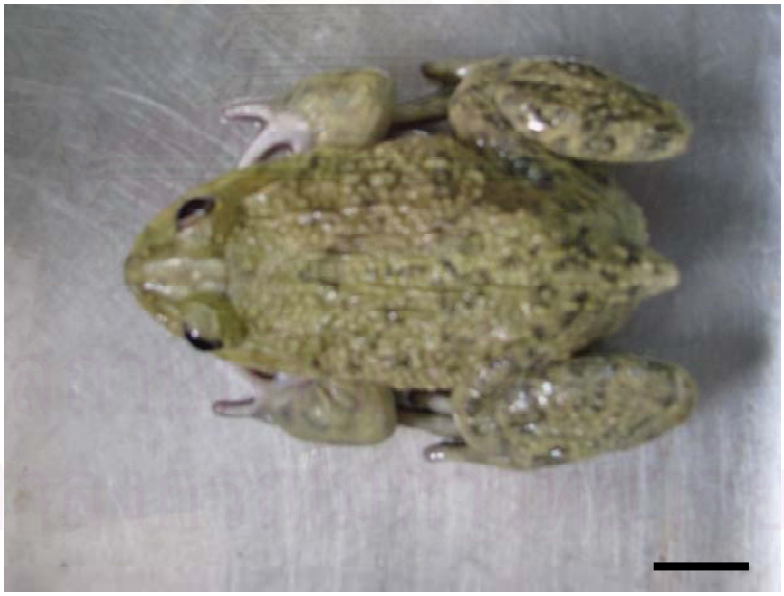
with the toes nearly full webbed, digits without discs, and the tips of toes slightly swollen, and no outer metatarsal tubercles. There are about ten rows of warts and ridges on the back and inter-orbital space which are much narrower than the upper eyelid (Taylor, 1962).

In the breeding season from March to September, male frogs show well developed vocal sacs, recognized by wrinkles and a black coloring. The arms usually have black markings called nuptial pads for amplexus. In adult females, there are roughening and a reddening at the abdomen in preparation for male amplexus and the abdomen enlarged with eggs.

At present, this frog has been cultivated throughout Thailand for live stock purpose. It can be reared and reproduced (both under natural and hormone stimulated condition) in captivity. Moreover, this frog is considered as a high potential agricultural resource both for domestic markets and export. The export data in 2007-2008 reported 12,607 tons for a value of 99.93 million baht and this demand trend is increasing (Ministry of Commerce Thailand, 2008).

Amphibians especially anuran have been extensively used for scientific research and this frog species *H. rugulosus* can be readily reared and breed in captivity. Therefore, *H. rugulosus* could be considered as a potentially anuran model in the region of its distribution, South East Asia.

a



b

Figure 1-4: Adult *Hoplobatrachus rugulosus* (a) female and (b) male frogs

(bar= 2 cm)

CHEPTER II

LITERATURE REVIEW: TOXICITY AND ECOTOXICOLOGY PROFILES OF ATRAZINE

The routes of entry of atrazine are oral, dermal, and inhalation (Dorsey *et al.*, 2003). The potentially effected organisms are those that live in the areas where atrazine was applied at high levels (U.S. EPA, 2005). Moreover, the effected organisms may be exposed to a low level of atrazine contamination both in the atmosphere and in drinking water (Dorsey *et al.*, 2003). The U.S. EPA 'National Survey of Pesticides in Drinking Water Wells' found that atrazine was the second most frequently detected herbicide (U.S. EPA, 2005). The U.S. EPA Maximum Contaminant Level (MCL) of atrazine allows in drinking water was 3 ppb and the Thai authorities also use the same control level (U.S. EPA, 2005; Thailand Pollution Control Department, 2000).

Atrazine is considered to be an endocrine disruptor, which is defined as exogenous substances that act like hormones in the endocrine system and disrupting the physiological functions of endogenous hormones. The primary effects of atrazine on organisms after exposure were on the reproductive and developmental processes (Dorsey *et al.*, 2003). In experimental animals atrazine could alter the estrus cycle in Sprague-Dawley, Long-Evans, and Donryu rats following exposure to 5 mg/kg/day atrazine for intermediate or chronic durations (Aso *et al.*, 2000; Cooper *et al.*, 1996; Eldridge *et al.*, 1994; 1999) or to a single dose of 300 mg/kg/day (Cooper *et al.*, 2000). It has been reported that it could induce mammary tumor formation in female Sprague-Dawley rats, but not male Sprague-Dawley or male or female F344 rats (Stevens *et al.*, 1994; 1999; Wetzel *et al.* 1994). Atrazine has also been shown to alter

serum luteinizing hormone (LH) and prolactin levels in Sprague-Dawley rats by altering the hypothalamic control of these hormones (Cooper *et al.*, 2000). However, no data are currently available regarding this mechanism in humans.

In summary, the U.S. EPA in 2005 summarized the toxicological profiles of atrazine in stating that atrazine could cause attenuation of the LH surge, decreases in pituitary hormone levels, ovarian histopathological effects (changes in ovarian tissue), and liver effects including increased serum lipids and liver enzymes, and liver histopathological effects, other effects on the central nervous system, immune system, and cardiovascular functions (U.S. EPA, 2005).

The U.S. EPA also published the 'Carcinogenicity Hazard Assessment and Characterization' that classified atrazine as 'not classifiable' due to insufficient evidence for carcinogenicity. The World Health Organization International Agency for Research on Cancer (IARC) classifies atrazine as not classifiable (Group 3) regarding carcinogenicity in humans.

The ecotoxicological studies of atrazine have also been conducted on many organisms. In invertebrates, for chronic effect studies, it was found that insects and crustaceans growth stages and/or number of young were reduced by atrazine exposures (U.S. EPA, 2006). Acute toxicity data for eight freshwater invertebrate species ranged from 3 ppm for the hydroid coelenterate, *Hydra* sp. (Brooke, 1990) to 49 ppm for the cladoceran, *Daphnia magna* (U.S. EPA, 2006). For stonefly (*Acroneuria* sp.) an EC_{50} of 6.7 ppm was reported (Brooke, 1990). The other invertebrate species tested, the snails (*Physa acuta* and *Physa* sp.) and an annelid (*Lumbriculus variegatus*), had LC_{50} values in excess of 20, 34.1 and 37.ppm, respectively (Roses *et al.* 1999; Brooke 1990).

Freshwater field studies were also conducted to determine the effects of atrazine. For example, Kettle *et al.* (1987) studied the effects of atrazine on macrophytes and bluegill sunfish populations. The experimental study was performed in 0.045-hectare, 2.1-meter deep ponds with each pond containing 50 bluegills, 20 channel catfish and 7 gizzard shads and treated with 20 ppb atrazine. After 136-days results showed that there were no significant effects on the mortality of the originally stocked fish, but the number of young bluegills was significantly reduced and bluegills were the only species to spawn during the study. Moreover, atrazine may alter the dieting of adult bluegills. The macrophyte community in treated ponds was noticeably reduced relative to controls throughout the summer and treated ponds also had fewer species of macrophytes. The authors concluded that macrophyte communities were directly affected through inhibition from atrazine, with the bluegill young suffering from the effects of the impoverished diet.

The data range of acute freshwater fish LC_{50} values were 5.3 to 6 ppm, therefore atrazine is categorized as slightly to moderately toxic for freshwater fish (U.S. EPA, 2006). Chronic effects of atrazine on freshwater fish embryos in 86 day treated Rainbow trout (*Oncorhynchus mykiss*) found atrazine delayed hatching at 1.1 and 3.8 ppm and the mortality was 58.8% at 3.8 ppm (U.S. EPA, 2006). In addition, U.S. EPA (2006) also reported the effects of atrazine in rainbow trout and catfish embryo-larvae reared in hard water (high mineral content water) and concluded that atrazine was highly teratogenic in all tests. George and Nagel (1990) also reported effects of atrazine in zebrafish (*Brachydanio rerio*) and found a 2-3 % increase edema and 35 days LC_{50} was 890 ppb. Wieser and Gross, 2002 investigated adult largemouth bass (*Micropterus salmoides*) exposed to atrazine for 20 days. The results showed elevated plasma vitellogenin and significantly decreased plasma 11-ketotestosterone.

Treated fish had significantly increased in plasma estradiol in females and a significant decrease in 11-ketotestosterone in males. Atrazine was reported to alter the swimming behavior of 1-week exposed zebra fish (*Brachydanio rerio*) at concentrations of 5 to 3,125 ppb (Steinberg *et al.*, 1995). The authors suggest that atrazine at concentrations commonly found in surface water may have an effect on the sensory organs and the nervous system (U.S. EPA 2006).

Atrazine was reported to induce alterations in 10 cm rainbow trout after 28-days exposure with 5, 10 and 20 ppb, by affecting renal corpuscles, renal tubules, renal interstitiums, and glomerular filtration by reducing Bowman's space due to podocyte proliferation (Fischer-Scherl *et al.*, 1991). Moreover, renal corpuscles appeared to be hypercellular and enlarged (*i.e*., hypertrophy) due to a proliferation of podocytes and mesangial cells at higher chronic concentrations (40 and 80 ppb) (Fischer-Scherl *et al.*, 1991). Furthermore, proximal and distal tubular epithelia showed lysis of the cytoplasm. Formation of vacuoles and vesicles and condensation of mitochondria was also prominent at 80 ppb.

Further experiments in three fish species; *Oncorhynchus mykiss*, *Galaxias maculatus*, and *Pseudaphritis urvillii* exposed to atrazine for 10 days demonstrated statistically significant ($p < 0.05$) effects occurred at levels 0.9 and 3.0 ppb such as the significantly reduced DNA levels in *Galaxias maculatus* at 10 ppb, reduction in glutathione (GSH) in the liver of *Pseudaphritis urvillii* at 50 ppb and reduction of protein levels in muscle *Oncorhynchus mykiss* (Davies *et al.*, 1994). Alazemi *et al.* (1996) reported that gill epithelium was damaged at 500 ppb atrazine in mormyrid fish, *Gnathonemus petersii*.

The study of two Nile River fishes (*Oreochromis niloticus* and *Chrysichthyes auratus*) exposed to 3 and 6 ppm atrazine for up to 28 days by Hussein *et al.* (1996)

showed several indicators of toxicity such as rapid respiration and increased rate of gill cover movements; slower reflexes and swimming movements; reduction in feeding activities; and loss of equilibrium leading to death. Several enzyme activity levels in serum and in some organs were changed by atrazine at concentrations of 1.5, 3 and 6 ppm in carp *Cyprinus carpio* L. (Neskovic *et al.*, 1993). There was a significant drop in alkaline phosphatase activity in the liver and no statistically significant effects were found on glutamic-pyruvic transaminase ($p<0.01$). Histopathological studies exhibited damage to gills ($\geq$1.5 ppm), liver (almost normal at 1.5 ppm) and vacuolization of hepatocytes at $\geq$3 ppm and intestine (slightly greater lymphocyte infiltration and stronger mucous secretion at 6 ppm). In Atlantic salmon (*Salmo salar* L.), atrazine also effects olfactory functions (nominal concentrations of 0.5, 5, 10, and 20 ppb atrazine) (Moore and Waring, 1998). They also reported effects in 7 days exposed Atlantic salmon smolts in fresh water, with significantly reduced gill Na^+K^+ATPase activity at concentrations of 2.0, 5.0 and 10.0 ppb. After that, fish were transferred to sea water and exposed to atrazine; a sea water challenge caused mortalities in smolts that had been pre-exposed to atrazine in fresh water at concentrations of 1.0, 2.0, 5.0, 10.0 and 22.7 ppb compared with control. Moreover, surviving fish showed signs of major physiological stress such as elevated plasma cortisol, thyroxine, osmolality, and monovalent ion concentrations.

Acute exposures by atrazine in amphibians were high and chronic mortality data for amphibians confirms that exposure to atrazine does not cause direct mortality to frogs and salamanders (U.S. EPA, 2006). In bullfrogs (*Rana catesbiana*) and American toads (*Bufo americanus*) embryos exposed to atrazine were studied by Birge *et al.* (1983). The 4 days LC_{50} was 410 ppb and more than 4,800 ppb, respectively. The abnormalities were defects of the head and vertebral column,

dwarfed bodies, partial twining, microcephaly, absent or reduced eyes and fins, and amphiarthrodic jaws. Although an LC_{50} of 410 ppb was reported in bullfrogs, 92% survival was observed at 410 ppb in the study. Survival did not fall below 50% until atrazine concentrations exceeded 15,000 ppb. Therefore, there is considerable uncertainty in the LC_{50} reported by Birge *et al.* (1983) of 410 ppb (U.S. EPA, 2006).

Amphibians mortality studies conducted by Allran and Karasov (2001) reported 14 day acute LC_{50} of more than 20 ppm in three frogs, leopard frog (*Rana pipiens*), wood frog (*Rana sylvaticas*), and American toad (*Bufo americanus*). Based on these values, the amphibians evaluated are relatively insensitive to atrazine on an acute exposure basis (U.S. EPA, 2006). However, the effects of sub-lethal concentrations were observed at 4.3 ppm and higher. The sub-lethal abnormalities were elevated ventilation rates (4.3 ppm and higher) and reduced feeding (20 ppm only) in adults and increased incidences of deformities in survivors at 4.3 ppm and higher. The deformations were wavy tails, lateral tail flexures, facial edemas, axial shortenings, dorsal tail flexures and blistering observed for all species tested.

The four species of tadpole frogs including spring peepers (*Pseudacris crucifer*), American toads (*Bufo americanus*), green frogs (*Rana clamitans*), and wood frogs (*Rana sylvatica*) were studied by Storrs and Kiesecker (2004). The embryos were exposed to nominal atrazine concentrations 3, 30, and 100 ppb at different stages of development *i.e.* early and late larvae stages. The results showed no significant differences in survivorship between the treatment levels and the controls were observed for late spring peepers, early toads, and late wood frogs. However, with late stages of the toad and wood frog, there was significantly lower survival for animals exposed to 3 ppb as compared with the higher treatment groups. Significant

differences in survivorship within the 3 ppb treatment group relative to the control were observed for late stages of the toad and both stages of the green frog.

Sub-lethal effects of atrazine were studied in various frog species, for example, the U.S. EPA (2006) reported the exposure of three frog species *i.e.*, cricket frogs (*Acris crepitans*), wood frogs (*Rana sylvatica*), northern leopard frogs (*R. pipiens*) to technical grade atrazine at concentrations ranging from 30 to 600 ppb from the first feeding stage through to metamorphosis. *A. crepitans* manifested delayed development and reduced post metamorphic dry body weight at 300 ppb atrazine. However, no effects were found on the other two frog species tested (*R. sylvatica* and *R. pipiens*).

In a microcosm investigation by Diana *et al.* (2000) on larval gray tree frogs (*Hyla versicolor*) in artificial 1.22-m diameter ponds containing phytoplankton, periphyton, and the aquatic macrophyte, marshpepper knotweed (*Polygonum hydropiper*). The nominal concentrations of atrazine were 0, 20, 200, and 2000 ppb. Over the course of the 40 days study, chlorophyll *a* was lowest in the control, highest in 200 ppb, and intermediate in 20 and 2000 ppb groups. Macrophyte biomass at the end of the study decreased correlating with the concentration of atrazine. Frogs from the two higher treatment groups were statistically shorter and had lower body weight at metamorphosis (10% reduction) than those from the control and low atrazine groups. Time to metamorphosis was 5% longer in the 2000 ppb groups than in the 200 ppb group, but did not differ statistically from controls in any treatment group. However, no difference in length or body mass at metamorphosis was detectable and no significant treatment-related differences were detected for survival rates.

Boone and James (2003) studied the post-application effects of atrazine on body mass development, and the survival of two anuran species, the southern leopard

frog, (*Rana sphenocephala*) and the American toad (*Bufo americanus*) and two caudate species, Spotted salamander (*Ambystoma maculatum*) and Small-mouthed salamander (*A. texanum*). The animals were reared in outdoor mesocosms containing leaf litter and plankton from natural ponds. Atrazine was added at only one concentration of 200 ppb. The results showed that atrazine reduced chlorophyll concentration of algal communities and resulted in reduced mass for toads and leopard frogs and lengthened larval periods for small-mouthed salamanders. The authors suggested that atrazine reduced the food supply of leopard frog tadpoles to some extent and increased starvation resulting in abnormal development. In contrast, the studies on salamander were also conducted by Rohr *et al.* (2003) in embryos and larvae of streamside salamander *A. barbouri* for 37 days at nominal concentrations of 4, 40, and 400 ppb in the presence and absence of food. The results showed no effect on embryo or larval survival, hatching, or growth rates at any of the test concentrations. Rohr *et al.* (2004) also studied the combined effects of food limitation and drying conditions on the survival, behavior, and metamorphosis of the streamside salamander from embryo stage through metamorphosis at nominal atrazine concentrations of 4, 40 and 400 ppb. The results showed that drying conditions and food limitation decreased larval survival, while 400 ppb atrazine only reduced larval survival in one of the two years tested. Drying conditions accelerated metamorphosis for larvae exposed to 0 and 4 ppb atrazine, but did not affect metamorphosis timing for the 40 or 400 ppb groups. The authors concluded that food limitation, drying conditions, and 400 ppb of atrazine reduced size at metamorphosis without affecting body condition.

In reptiles, the endocrine effects of atrazine on embryonic sexual differentiation were performed in the turtle, red-eared Slider *Pseudemys elegans* eggs and the American alligator *Alligator mississippiensis* eggs shortly after laying (U.S. EPA, 2006). Neither adverse effects nor the presence of atrazine in embryonic fluids were found at all concentration (0, 10, 50 100 and 500 ppb for 10 days). Thus, atrazine does not appear to penetrate the leathery shell of reptile eggs under these conditions (U.S. EPA 2006). In contrast, De Solla *et al.* (2005) conducted experiments on the same eggs incubated in soil treated with atrazine (the low and high atrazine treatment groups were 0.64 and 8.1 ppm) at the incubation temperature 25 ºC which normally produced only males. The results showed male gonad abnormalities which in some males gave testicular oocytes and females in atrazine treated groups but no such abnormalities could be found in the control group. However, there were no statistical differences found between the treated and control groups and the authors suggested that natural and spontaneous intersexes exist in turtle populations. In American alligator (*Alligator mississippiensis*) eggs exposed to atrazine at concentrations of 0, 0.014, 0.14, 1.4, and 14 ppm produced neither gonadal abnormalities nor reproductive tract histopathology, and no significantly difference of hepatic aromatase activity compared with the control (Crain *et al.*, 1999).

Regarding avian acute toxicology, the LC_{50} data were high (960 mg/kg) in northern bobwhite quail (*Colinus virginianus*), more than 2,000 mg/kg in mallard duck (*Anas platyrhynchos*) and ring-necked pheasant (*Phasianus colchicus*), and 4,237 mg/kg in Japanese quail (*Coturnix c. japonica*). Thus atrazine is categorized as slightly toxic to avian species based on acute oral exposures (U.S. EPA, 2006).

Moreover, the Avian Subacute Dietary Toxicity report found that the LC_{50} was more than 5,000 mg/kg (in Northern bobwhite, ring-necked pheasant, Japanese quail, and mallard duck), therefore, atrazine is categorized as practically nontoxic to avian species on a subacute dietary exposure basis (U.S. EPA, 2006). Other avian chronic studies were also conducted, resulting in reduced egg production and embryo viability/hatchability in experimental northern bobwhite quail and mallard duck (LOAEC and NOAEC values of 675 and 225 ppm, respectively) (U.S. EPA, 2006). Wilhelms *et al.* (2005) also reported that atrazine at 1,000 ppm reduced food consumption (15% reduction compared with controls) and weight gain (31% reduction), and elevated testosterone levels (approximately 3-folds increase) in male Japanese quail and also reported similar effects in females.

Table 2-1: Freshwater invertebrate acute toxicity (modified from U.S. EPA, 2006)

Surrogate Species/ Static or Flow-through	96-hour LC_{50}/EC_{50} µg/L (ppb) (measured/nominal)	Toxicity Category	Author,Year
Midge (*Chironomus tentans*) Static test	720 (nominal)	highly toxic	Macek *et al*., 1976
Midge (*Chironomus riparius*)	1,000 (unknown)	highly toxic	Johnson, 1986
Waterflea (*Daphnia magna*)	3,500 (unknown)	moderately toxic	Johnson , 1986
Waterflea < 24-hours old (*Daphnia magna*) 26-Hour static test	3,600 (unknown)	at least moderately toxic	Frear & Boyd,1967
Waterflea (*Ceriodaphnia dubia*) 48-Hour static test	> 4,900 (measured)	unknown	Jop, 1991
Scud (*Gammarus fasciatus*) Static test	5,700 (nominal)	moderately toxic	Macek *et al*., 1976
Stonefly (nymph) (*Acroneuria* sp.) Flow-through test 67.4 mg/L CaCO3	6,700 (measured)	moderately toxic	Brooke, 1990
Waterflea (*Daphnia magna*) Static test	6,900 (nominal)	moderately toxic	Macek *et al*., 1976
Scud juvenile (*Hyalella azteca*) Flow-through test 67.4 mg/L Ca CO3	14,700 (measured)	slightly toxic	Brooke, 1990
Scud juvenile (*Gammarus pulex*) Static-renewal - daily	14,900 (measured)	slightly toxic	Taylor, Maund & Pascoe, 1991
Leech (*Glossiphonia complanata*) Static-renewal weekly	> 16,000 (measured) 6,300 µg/L at 28 days	slightly toxic	Streit & Peter, 1978
Leech (*Helobdella stagnalis*) Static-renewal weekly	> 16,000 (measured) 9,900 µg/L at 27 days	slightly toxic	Streit & Peter, 1978
Snail (*Ancylus fluviatilis*) Static-renewal weekly	>16,000 (measured) > 16, 000 µg/L at 40 days (35 % mortality)	slightly toxic	Oris, Winner & Moore, 1991
Waterflea <12 hr old (*Ceriodaphnia dubia*) Static 48-hour test 57 mg/L CaCO3	> 30,000 (measured) Slope - no data	slightly toxic	Taylor, Maund & Pascoe, 1991
Midge (*Chironomus riparius*) Static-renewal - daily 10-Day test	> 33,000 (measured) 18,900 µg/L at 10 days	slightly toxic	Drake, 1976;Putt, 2002
Midge (*Chironomus tentans*) Flow-through 10-Day test; water-spiked exposure	Mortality: LC_{50} > 24,000 (measured) (37% mortality)	slightly toxic	
Waterflea (*Daphnia magna*) Flow-through test	49,000 (higher concs. than 31,000 µg/L were cloudy) (measured) slope 2.433	slightly toxic	Putt, 1991
Waterflea (*Daphnia pulex*) Static test; 15EC 282 mg/L hardness with and without sediment	36,500 (nominal) 46,500 (with sediment)	slightly toxic	Hartman & Martin, 1985

Table 2-2: Freshwater fish acute toxicity (modified from U.S. EPA, 2006)

Surrogate Species/ Static or Flow-through test	96-hour LC50 (ppb) (measured/nominal)	Toxicity Category	Author/Year
Rainbow trout (*Oncorhynchus mykiss*) Static test	5,300 (nominal)	moderately toxic	Beliles & Scott 1965
Brook trout (*Salvelinus tontinalis*) Flow-through test	6,300-4,900	moderately toxic	Macek *et al*. 1976
Fish from the Nile River *Chrysichthyes auratus* Static-renewal - daily 150 mg/L $CaCO_3$; 22ºC	6,370	moderately toxic	Hussein, El-Nasser & Ahmed 1996
Bluegill sunfish (*Lepomis macrochirus*) Flow-through test	> 8,000 6,700 (7-day test)	moderately toxic	Macek *et al*. 1976
Tilapia 38 g (*Oreochromis niloticus*) Static-renewal - daily 150 mg/L $CaCO_3$: 22ºC	9,370	moderately toxic	Hussein, El-Nasser & Ahmed 1996
Fathead minnow (*Pimephales promelas*) 24-Hour renewal test	15,000 (nominal) 15,000 (5-day test)	slightly toxic	Macek *et al*. 1976
Carp (*Cyprinus carpio*) Semi-static test	18,800 (nominal)	slightly toxic	Neskovic *et al*. 1993
Fathead minnow juvenile (*Pimephales promelas*) Flow-through test; 52 mg/L $CaCO_3$	20,000 (measured)	slightly toxic	Dionne 1992
Bluegill sunfish (*Lepomis macrochirus*) Static test	24,000 (nominal)	slightly toxic	Beliles & Scott 1965
Brown trout (*Salmo trutta*) 1.9 g Static-Renewal - daily pH 6; 10EC; 11 mg/L $CaCO_3$ Zebrafish (*Brachydanio rerio*)	27,000 (nominal)	slightly toxic slightly toxic	Grande, Anderson & Berge 1994, NR Korte & Greim 1981
Bluegill sunfish (*Lepomis macrochirus*) Static test	57,000 (nominal)	slightly toxic	Buccafusco 1976
Goldfish (*Carassius auratus*) Static test	60,000 (nominal)	slightly toxic	Beliles & Scott 1965

Table 2-3: Amphibian mortality/survivorship toxicity tests

(modified from U.S. EPA, 2006)

Study type/ Test material	Test Organism (Common and Scientific Name) and Age and/or Size	Test Design	Endpoint Concentration in ppb	Author/ year
Acute lab (14 days) /	-Leopard frog (*Rana pipiens*) -Wood frog (*Rana sylvaticas*) -American toad (*Bufo americanus*)	- Renewal - Hardness (mg/L as $CaCO_3$) = 290 Target Temp: 22 °C Animals were exposed in the embryonic stage.	LC_{50} for all 3 species= >20,000 (measured). Effects included increased incidence of deformities in embryos exposed for 4 days after hatching and elevated ventilation rate in exposed adults at 4.3 mg/L and higher.	Allran and Karasov, 2001
Chronic (32 d) lab study / Atrazine commercial-grade	- Spring peeper (*Pseudacris crucifer*) -American toad (*Bufo americanus*) -Green frog (*Rana clamitans*) -Wood frog (*Rana sylvatica*) -All tadpoles at early (Gosner stages 25-27) and late (stages 29-36) developmental stages	- Static renewal (water replaced every 3 d) at nominal concentrations of 0, 3, 30, and 100 ppb. -Measured conc (after 1 d = ND, 2.84, 25.2, and 64.8 ppb) - Peepers, toads, and early-stage green frogs kept in 120 ml polypropylene cups w/100 ml (treatment in dechlorinated water); late wood frog and green frogs kept in 750 ml poly cups w/ 500 ml water; tadpoles/treatment varied - Temperature = 22 ºC - Photoperiod = 12 h light/dark - Feeding: crushed alfalfa every 3 d - Endpoints: Surivorship	Early spring peeper: LOAEL = 64.8; NOAEL = 25.2 Late spring peeper: NOAEL = 64.8 Early A. toad: NOAEL = 64.8 Late A. toad: LOAEL = 2.84 NOAEL = <2.84 Early green frog: LOAEL = 2.84 NOAEL = <2.84 Late green frog: LOAEL = 2.84 NOAEL = <2.84 Late wood frog: NOAEL = 64.8	Storrs and Kiesecker, 2004
Acute, developmental study; Atrazine technical unspecified purity	Bullfrog and American toad embryos	Eggs were exposed from fertilization to 4 days post hatch. Atz Concs: 28 to 4800 ug/L Exposure: flow through Endpoints: Presence of gross debilitating anomalies. Temp: 12-14 DegC pH: 7 – 7.8	Bullfrog LC_{50}: 410 ug/L American toad LC_{50}: >4800 ug/L	Birge et al., 1983.

Table 2-4: Reptilian toxicity tests (modified from U.S. EPA, 2006)

Study type/ Test material	Test Organism (Common and Scientific Name) and Age and/or Size	Test Design	Endpoint Concentration in ppm	Author/ year
Chronic lab (117 days) / Atrazine 480 formulation (atrazine content = 481 g/L and unspecified triazines of 29 g/L)	Snapping turtle (*Chelydra serpentine*) eggs	- Eggs incubated in soil treated w/atrazine at 1.32 lb ai/A (measured conc = 0.64 ppm) and 13.2 lb ai/A (measured conc = 8.1 ppm) and control. - 3 replicates (with 23-24 eggs/replication)/treatment group. - Incubator temp = 25o (+1oC) to produce males. - Endpoints: gonadal development (hatching success, gonadal morphology, and thyroid activity)	NOAEC = 13.2 lb ai/A (0.81 ppm) Some males w/testicular oocytes and females produced in atrazine-treated groups (3.3 – 3.7%); however, no significant differences between atrazine treatments and controls were observed. Thyroids from each treatment and control displayed similar levels of activity.	De Solla *et al.*, 2005
Chronic lab (duration NR) / Atrazine (99 % ai)	American alligator (*Alligator mississippiensis*) eggs at stage 21 in embryonic development, just prior to onset of gonadal differentiation	- Eggs were treated w/atrazine at 0, 0.014, 0.14, 1.4, and 14 ppm via topical application to the eggshell in 50 µl of 95% ethanol. - 5 eggs/treatment were incubated at temperatures to produce either 100% males (33 ºC) or 100% females (30 °C). - Endpoints: gonadal histology and hepatic steroidogenic activity	NOAEC = 14 ppm All atrazine treated eggs incubated at female- and male-determining temps produced female and male hatchlings, respectively. No differences in gonadal histology (Mullerian duct epithelial cell height and medullary regression) and hepatic aromatase activity was noted between atrazine treated groups and controls.	Crain *et al.*, 1999

Table 2-5: Avian acute oral toxicity (modified from U.S. EPA, 2006)

Surrogate Species	LD_{50} (mg/kg)	Toxicity Category	Author/Year
Northern bobwhite quail (*Colinus virginianus*) 14-day old chicks; 8-day test	940	Slightly toxic	Fink, 1976
Mallard Duck (*Anas platyrhynchos*) 6-months old; 14-day test	> 2,000	Practically non-toxic	Hudson, Tucker & Haegle, 1984
Ring-necked Pheasant (*Phasianus colchicus*) 3-months old; 14-day test	> 2,000	Practically non-toxic	Hudson, Tucker & Haegle,1984
Japanese Quail (*Coturnix c. japonica*) 50-60 days old; 14-day test	4,237	Practically non-toxic	Sachsse and Ullman, 1974

CHAPTER III

THE NORMAL DEVELOPMENT OF RICE FIELD FROG

Hoplobratrachus rugulosus (Weigmann, 1834)

Introduction

The anuran embryo is considered as one of the more suitable laboratory animals which is appropriate as a model in embryological and developmental biology studies. Its aquatic embryos and larvae can easily be reared in the laboratory and adults can be stimulated to reproduce in captivity after hormone or hormone analogue induction (Nieuwkoop and Faber, 1999). Moreover, the *ex vivo* embryonic development has also facilitated the investigative process.

The study of anuran embryology is facilitated by the use of staging tables (Gosner, 1960). Staging is the identification of specific morphological landmarks that appear useful in comparing the sequence of events in a developmental continuum (McDiarmid and Altig, 1999). These tables are indispensible to many studies covering the frog life cycle (Gosner, 1960), embryology, developmental biology and has gained considerable merit from biologists in other fields (Weisz, 1945). With the use of a staging system, tadpoles of different species which are widely disparate in size and developmental period but have completed similar developmental steps can be compared (McDiarmid and Altig, 1999). It is not possible, however, to apply a system to all taxa under all conditions because the appearance of morphological structures at different temperatures varies within taxa.

The studies of the staging of anuran have been broadly defined by at least two categories: the external morphology and the age at constant temperature (Pollister and Moore, 1937). In embryonic stages, just after fertilization up to the growth of the tail, the changes of external morphology are relatively simple (Pollister and Moore, 1937), making it easier to indentify certain developmental stages, such as cleavage, gastrulation and neurulation. In contrast, there are many simultaneous processes in the later periods of embryonic development. Therefore, certain physiological evidence has been applied to facilitate the stages identification. The most obvious characteristics are: growth of the tail, elongation of the body, growth of external gills, development of the operculum, development of transparency of the epidermis, coiling of the gut. Furthermore, the development of certain physiological features e.g. beginning of muscular contraction, heart beating, gill circulation, tail fin circulation and hatching of the embryo, including beginning of swimming ability have also been used (Pollister and Moore, 1937). The length of embryos and the ratio of some parts of larvae have also been widely used as a basis for definition. In wider practice, however, the total length of embryo is not very reliable because of the egg size variability, both intra-specifically and inter-specifically. Data in total length are, nevertheless, included in the table (Pollister and Moore, 1937).

There are many staging tables which have been published for a variety of anuran. More than 45 staging tables for anuran were produced, however, only four of them are most cited: Gosner 1960, general; Nieuwkoop and Faber 1956, 1967, *Xenopus*; Shumway 1940, *Rana*; and Taylor and Kollros 1946, *Rana* (McDiarmid and Altig, 1999).

The tables for the normal development of *Rana sylvatica* were tabulated by Shumway 1940. Eggs were collected from both natural habitat and laboratory condition. The data from this experiment shown no significant difference in the development of fertilized eggs obtained by both methods, which correspond well with the previous report of Pollister and Moore (1937) who worked on *Rana pipiens*. Shumway tabulated twenty-five stages at 18^{o} C based on Pollister and Moore (1937) with some modifications. This table is less complete in some cases but applicable for several other species (Gosner, 1960). The table which Pollister and Moore developed is composed of twenty-five Arabic numeral pre-feeding stages, from fertilization until the completion of operculum development. However, they have not reported any data for later developmental stages after operculum development until the end of metamorphosis.

The study of Taylor and Kollos in 1946 reported normal development stages of post-feeding *Rana pipiens* larvae. The study was conducted at 20^{o} C and tabulated twenty-five stages with Roman numerals from I to XXV. This table is also available for several other species. However, the numeral system in this study does not correspond with the embryo series by Shumway (Gosner, 1960).

One of the most commonly cited staging has been developed by Nieuwkoop and Faber in 1956 when *Xenopus laevis* became a common laboratory animal model (Nieuwkoop and Faber, 1999). The normal table of *Xenopus laevis* was tabulated into Arabic numerals from early development up to the end of metamorphosis. This staging is considered to be of the highest developmental resolution which is composed of both morphological and anatomical descriptions. This staging could be useful for experimental studies in embryology, developmental biology and other biological studies using anuran as the model animal.

A simplified table for staging anuran embryo and larvae was produced by Gosner in 1960, who tries to simplify the staging table making it adequate for "generalized" developmental series (Gosner, 1960). This system composed of 46 Arabic numeral stages which combined and modified twenty-five pre-feeding stages by Shumway (1940) and twenty-five post-feeding stages by Taylor and Kollros (1946). This system was reported to work reasonably well and is regarded as the one that meets the prime objective in allowing inter-specific comparisons (McDiarmid and Altig, 1999) as compared with the system from Nieuwkoop and Faber (1956) which contained more high developmental stage resolution within short developmental sequences.

There is a limited amount of embryological data for the rice field frog. Pariyanont *et al.* in 1990 reported the body length of 7, 24, 28, and 42 day of development compared with Taylor and Kollros 1946 stage. As mentioned in chapter I, the rice field frog, *Hoplobatrachus rugulosus*, is capable of being a potentially anuran model for scientific research in South East Asia. The rice field frog can be reared and induced to reproduce in captivity which has lead to frog farming in many areas in Thailand and also in several South East Asian countries where it is distributed. Several aspects of the reproductive biology and physiology of this frog have been investigated over the past decade, however there is limited information regarding the embryology and developmental biology.

Materials and Methods

Adult males and females *H. rugulosus* were obtained from the Huai Hong Krai Royal Development Study Centre, Chiang Mai province and also from the Amphibian and Reptile Research Unit, Chulalongkorn University, Bangkok. For adults rearing in the laboratory, mature male and female frogs were maintained separately in 500 liters plastic tanks containing de-chlorinated tap water and fed once a day with frog chow, Higrade™.

The adult frog which can be readily bred can be recognized by some well developed sexual characteristics. For the male frog, arms usually have black markings called nuptial pads and the well developed vocal sac. In the female frog, there is roughening and reddening at the abdomen making her ready to receive the male amplexus. Three pairs of male and female frog were used in this study.

Spawnings were induced by the intra-peritonial administration of human chorionic gonadrotrophin (GnRH) analogue, Suprefact™ (Buserlin) (Hauy Hong Krai Royal Development Study Centre, 2002) dissolved in 0.75% NaCl. The selected mature females received two GnRH analogue administrations; the first dose was 2 µg per kilogram body weight of GnRH analogue, 6 hours before mating. The second dose was 4 µg per kilogram body weight instantly before putting males and females together. Male frogs received 2 µg per kilogram body weight of GnRH analogue just prior to mating. Three pairs of male and female frogs were allowed to breed naturally in a 200 liter glass tank with 10-20 litres of de-chlorinated tap water and twigs were provided for oviposition.

Spawning occurred between 9 p.m. to 5 a.m. After spawning, the adults were taken out. Successful fertilization was checked by observing the presence of fertilization membranes using a dissecting microscope (Olympus® SZ230). Only batches of eggs with a high percentage of fertilization (more than 90%) were used for further study. The fertilized eggs were reared in 20 litre glass tank, 100 eggs per tank, in 4 litres 10% aerated Holfreter's solution. All tanks were kept at 25 ± 2 ºC with 12-hours/12-hours light dark cycle. The tanks were cleaned and the solution changed every 3 days, except in the first week. Feeding was started on day 3 after fertilization, larvae were fed with frog chow, Higrade™, *ad libitum*.

During the metamorphosis climax at the period of tail resorption, the larvae reduce their feeding and movements. In order to save them from drowning and being attacked by the less developed larvae, the tanks were slanted to provide some shallow areas and thin polyurethane plates were also added to permit the developing larvae to emerge from the water.

Normal development of *H. rugulosus* embryos and larvae were observed under the dissecting microscope (Olympus® SZ230). The normal table of 66 stages of *Xenopus laevis* of Nieuwkoop and Faber (1956) and 46 stages of Gosner (1960) were used as references in determining the developmental stages. The embryos and tadpoles that reached each stage were anesthetized and photographed, then 10 animals per stage were fixed with 10% normal buffered formalin for further study. This procedure describe in this study also in the next two chapters have been proved and allowed by Chulalongkorn University Animal Care and Use Committee (CU-ACUC 0723011)

Results

The air temperature throughout the experimental period ranged from 24.5-26.0 °C which resulted in the temperature of the Holfreter's solution used for raising the embryos and larvae ranged from 24.0-24.5 °C.

The results in this part were reported as; photographs of each developmental stage including total body length, time of each stage since fertilization (eggs collection) and all these data were summarized in table 2-1. The developmental stages were compared to amphibian development table conducted by Shumway (1940)-S, Taylor and Kollros (1946)-TK, Nieuwkoop and Faber (1956)-NF and Gosner (1960)-G stage.

Successful fertilization could be recognized by lifted up of the vitelline membrane which used to closely cross linked to the egg's cell membrane to become fertilization membrane. This fertilization membrane provided space for embryonic movement which cloud be clearly recognized in later embryonic stages (Figure 2-2 a). Another sign of successful fertilization was the presence of gray crescent (Figure 2-1 b) on one side of the egg near the equatorial plane of the egg as the result of cytoplasm rotation. The external dorsal (animal) side of amphibian egg usually covered with dark pigment, while cytoplasm of ventral (vegetal) side accumulated heavily yolk which resulted in the creamy color in contrast to the dorsal side. Cytoplasm rotation occurred after fertilization revealed lighter gray crescent in the area used to be covered with dark pigment. However, gray crescent could be recognized about ½ hour after fertilization, while fertilization membrane could be recognized about 1-1 ½ minutes after fertilization.

The first cleavage stage could be recognized by the presence of cleavage furrow cutting vertically across the fertilized egg resulted in embryo at 2 cells stage (Figure 2-2 a). The process took place around 60 minutes after fertilization and continued for about 90 minutes when completed 2 cells were formed (Figure 2-2 a). The second cleavage was also cleaved vertically given 4 cells embryo (Figure 2-2 b) and the process completed about 2 hours after fertilization. The third cleavage continued to cleave vertically gave rise to 8 cells embryo (Figure 2-3) at 2 hours 30 minutes. The fourth cleavage was the first horizontal cleaving resulted in 16 cells embryo (Figure 2-4 a and b). The fifth cleavage was along the vertical plane and completed at about 2 hours 45 minutes (Figure 2-4 c). From this stage onwards the embryo cleaved along vertical and horizontal plane alternatively. The cleaving embryo developed into morula stage with smaller cells or the “micromeres” found on the dorsal side (or animal hemisphere) while fewer and larger yolk filled cells, the “macromeres” were located on the ventral side (vegetal hemisphere) (Figure 2-4) .

The embryo proceeded to the early blastula and mid blastula (Figure 2-5) embryo could be recognized from external morphology by having more than 200 cells and boundary of each cell could still be clearly visible which made the external surface of the embryo appeared not smooth. However, when the embryonic development progressed to late blastula stage (Figure 2-6 a) with more cell divisions enabling to recognized this stage by smooth external surface of the embryos.

The gastrula stage could be recognized by the presence of lip of blastopore (Figure 2-6 b). The process began by pigmented cells of the animal hemisphere move downward (epiboly) to cover the vegetal yolk filled cells. Lip of blastopore was formed by invagination of cell at the line between pigmented cells and yolk filled

cells. The first lip of blastopore, “the dorsal lip of blastopore” was formed in the area that used to be the gray crescent. The cells from the animal hemisphere which moving downward then roll over the forming blastopore lip into the interior of the embryo by infolding process (involution). This gastrulation process could be recognized externally by the movement of the pigmented cells covering the yolky cells (Figure 2-6 b) The yolky cells covering the blastopore lip was called the “yolk plug”, which at the beginning appeared as large area and became progressively smaller (Figure 2-7). At mid-gastrula stage (Figure 2-7 a) the embryos rotated which made the blastopores or the yolk plugs which used to be at the vegetal pole became posterior pole of the embryos. As the process of epiboly and involution progressed the visible yolk plugs became smaller continuously until late gastrula stage the blastopore lips closed and yolk plugs were not visible. At this stage, the presumptive nervous system was visualized as pigmentation and thicken and flattening on the dorsal side of the embryos forming the neural plates.

The neurula stage were conspicuously identified by the presence of neural plate (Figure 2-8 a) which was formed by the thickening of the dorsal ectodermal epithelium. The folding up of the rims of neural plate leading the embryo to the neural fold stage (Figure 2-8 b). Closure of the neural folds forming the dorsal hollow neural tube along the dorsal midline of the embryo was now in neural tube stage (Figure 2-9 b). The embryo started to elongate anterior to posterior and at neural tube stage, the gill plates were indicated in the anterior region just below the neural tube (Figure 2-9 b)

After neural tube formation, the embryos were identified by the elongation of the tail bud (Figure 2-10). The organs formation process or organogeneses of the embryos were identified using certain physiological responses. At 1 day and 9hours

after fertilization, muscular contraction or muscular response of the embryos could be visualized (Figure 2-10 b). Blood circulation in gill areas and heart beat were visualized (Figure 2-11 a). The embryos hatching process started about 2 days after fertilization (Figure 2-11 b), newly hatch larvae had transparent cornea and tail fin circulation could be noticed (Figure 2-11 b).

By 4 days after fertilization, the larvae could be indicated by the fully developed operculum covering the gill which transformed the external gill to become internal gill (Figure 2-12 a). The formation of hind limb buds also started at this stage. The limb buds stages were identified using proportion between the length and the diameter of the developing limbs as criterion (Figure 2-12 b to f). After hind limb bud stage progress in formation of foot paddles were used to identify developing stages of the larvae (Figure 2-13). Proportional changes in the length of individual toes were also used as criterion at these stages.

The metamorphosis climax stages were identified by the emergence of fore limbs (Figure 2-14). The other important characteristics used at these stages were the progressive deepening of the mouth development as compare with the orbital space (Figure 2-14 to Figure 2-18). The end of metamorphosis was identified by the complete tail resorption and the larvae then became froglets. The total developmental period of *H. rugulosus* from fertilization to completed metamorphosis were 45±5 days at 25°C in laboratory conditions.

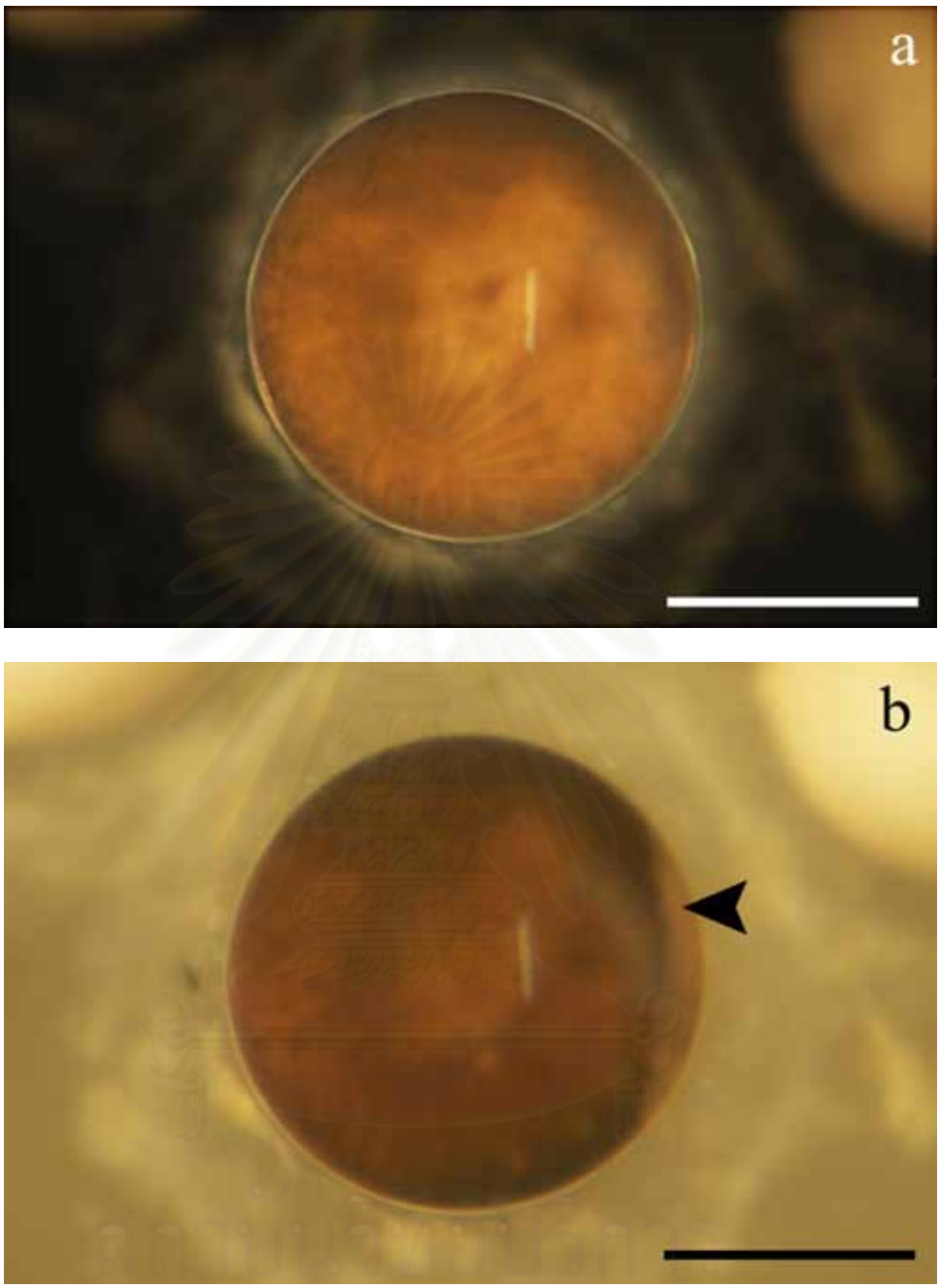


Figure 3-1: Photograph from the animal pole view *H. rugulosus* development (bar = 1 mm)

(a) The unfertilization egg

(b) Age 15-30 mins (after spawning): the appearance of the gray crescent (arrow head) after fertilization (Nieuwkoop and Faber stage 1, Gosner stage 2)

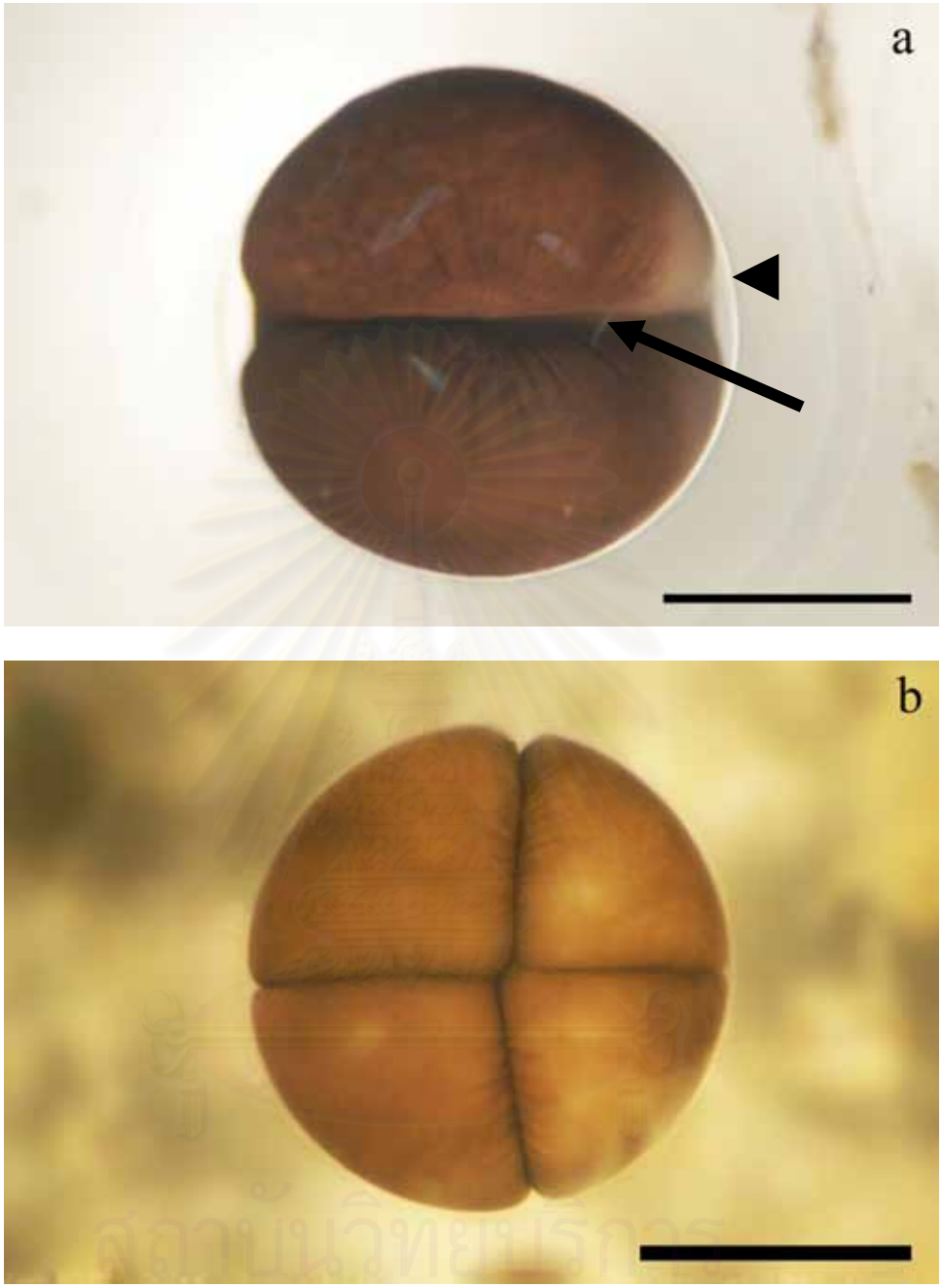


Figure 3-2: Photograph from the animal pole view *H. rugulosus* development (bar = 1 mm)

(a) Age 1 hr 30 mins: the completion of the first cleavage. Two cells stage. (Nieuwkoop and Faber stage 2, Gosner stage 3)

(b) Age 2 hrs: the completion of the second cleavage. Four cells stage. (Nieuwkoop and Faber stage 3, Gosner stage 4)

(arrow head-fertilization membrane, arrow-clevage furrow)

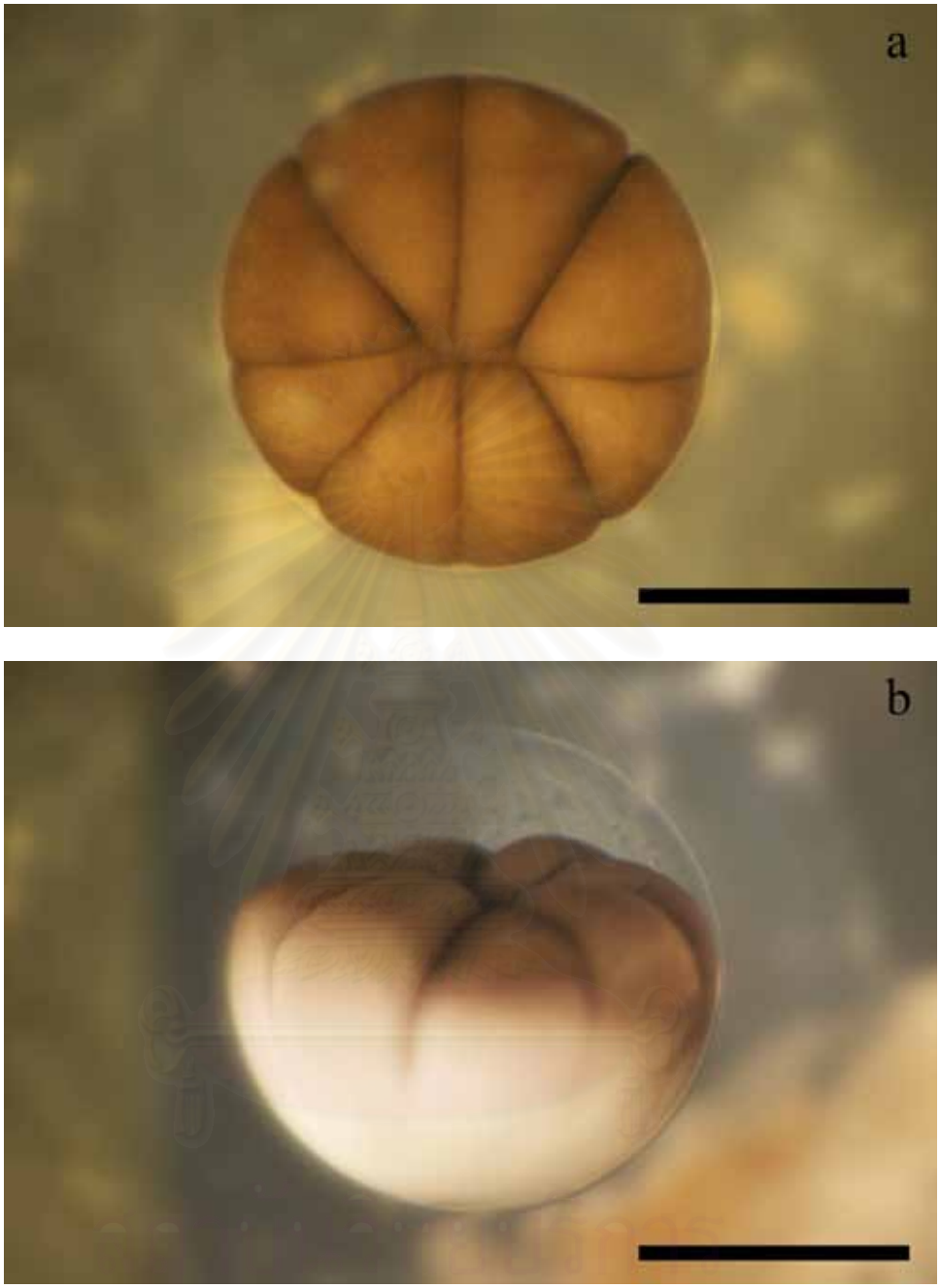


Figure 3-3: Photograph from (a) the animal pole and (b) dorso-lateral view of *H. rugulosus* development at age 2 hrs 15 mins, the completion of the third cleavage. Eight cells stage. (Nieuwkoop and Faber stage 4, Gosner stage 5) (bar = 1 mm).

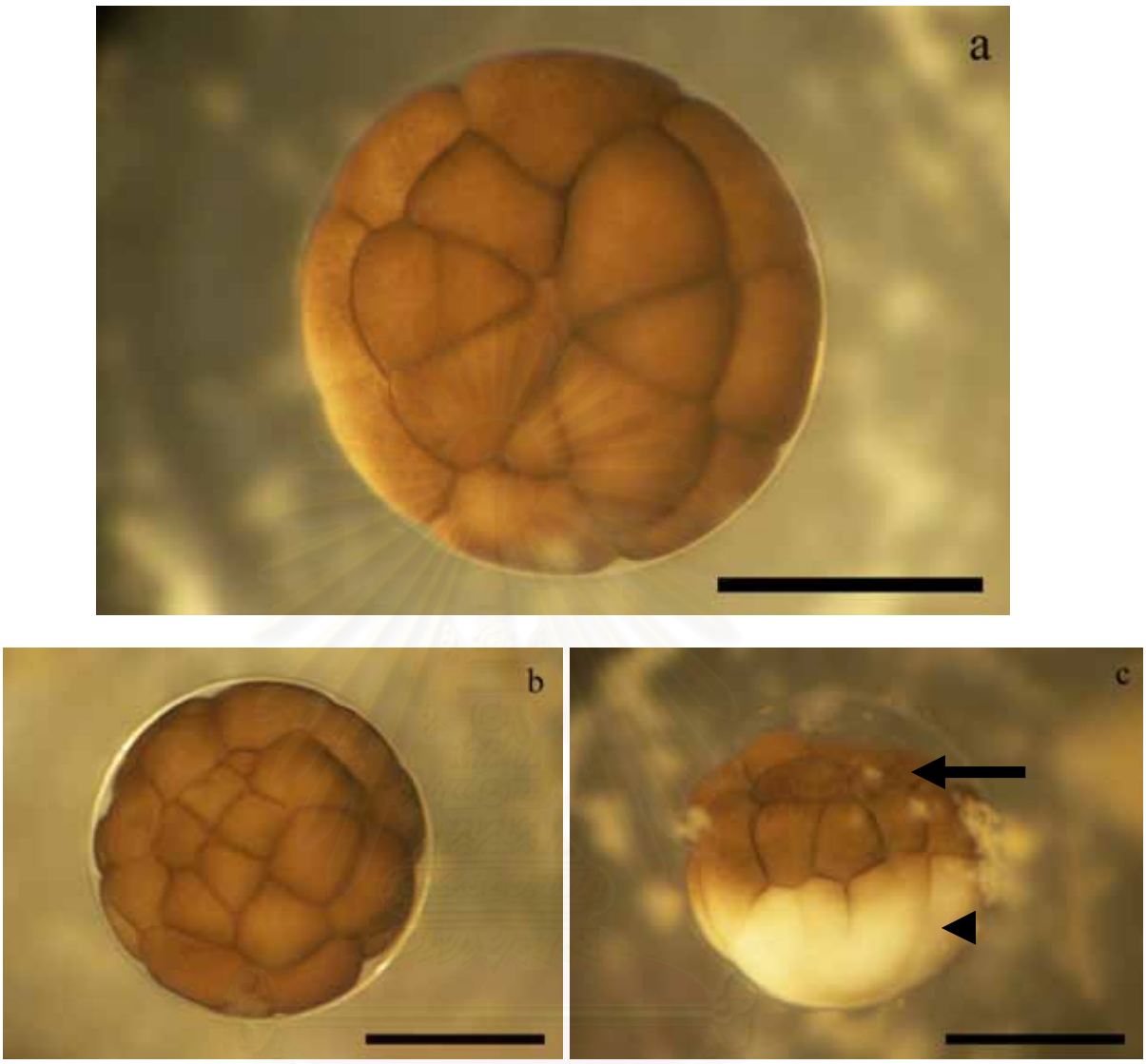


Figure 3-4: Photograph of *H. rugulosus* development (bar = 1 mm)

(a) Age 2 hrs 30 min: the completion of the fourth cleavage. Sixteen cells stage. (Nieuwkoop and Faber stage 5, Gosner stage 6)

(b) Animal pole view and-

(c) dorso ventral view of age 2 hrs 45 min: completion of the fifth cleavage. Thirty-two cells stage. (Nieuwkoop and Faber stage 6, Gosner stage 7) (arrow-micromeres, arrowhead-macromeres)

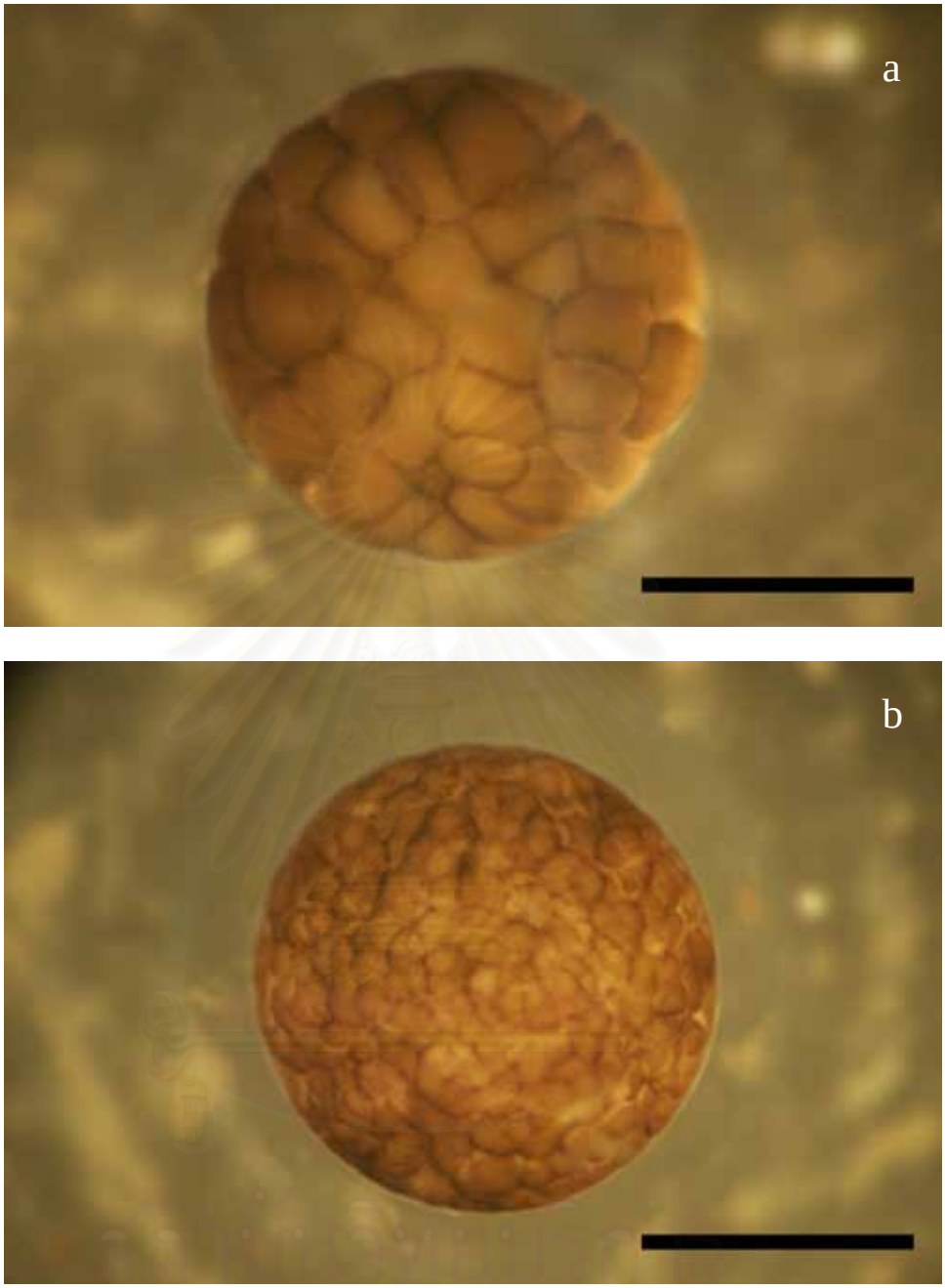


Figure 3-5: Photograph from the animal pole view *H. rugulosus* development (bar = 1 mm)

(a) Age 4 hrs 45 min: morula stage. (Nieuwkoop and Faber stage 7)

(b) Age 5 hrs: mid-blastula. The medium cell size macromeres as indicated, the surface not yet entirely smooth. (Nieuwkoop and Faber stage 7, Gosner stage 8)

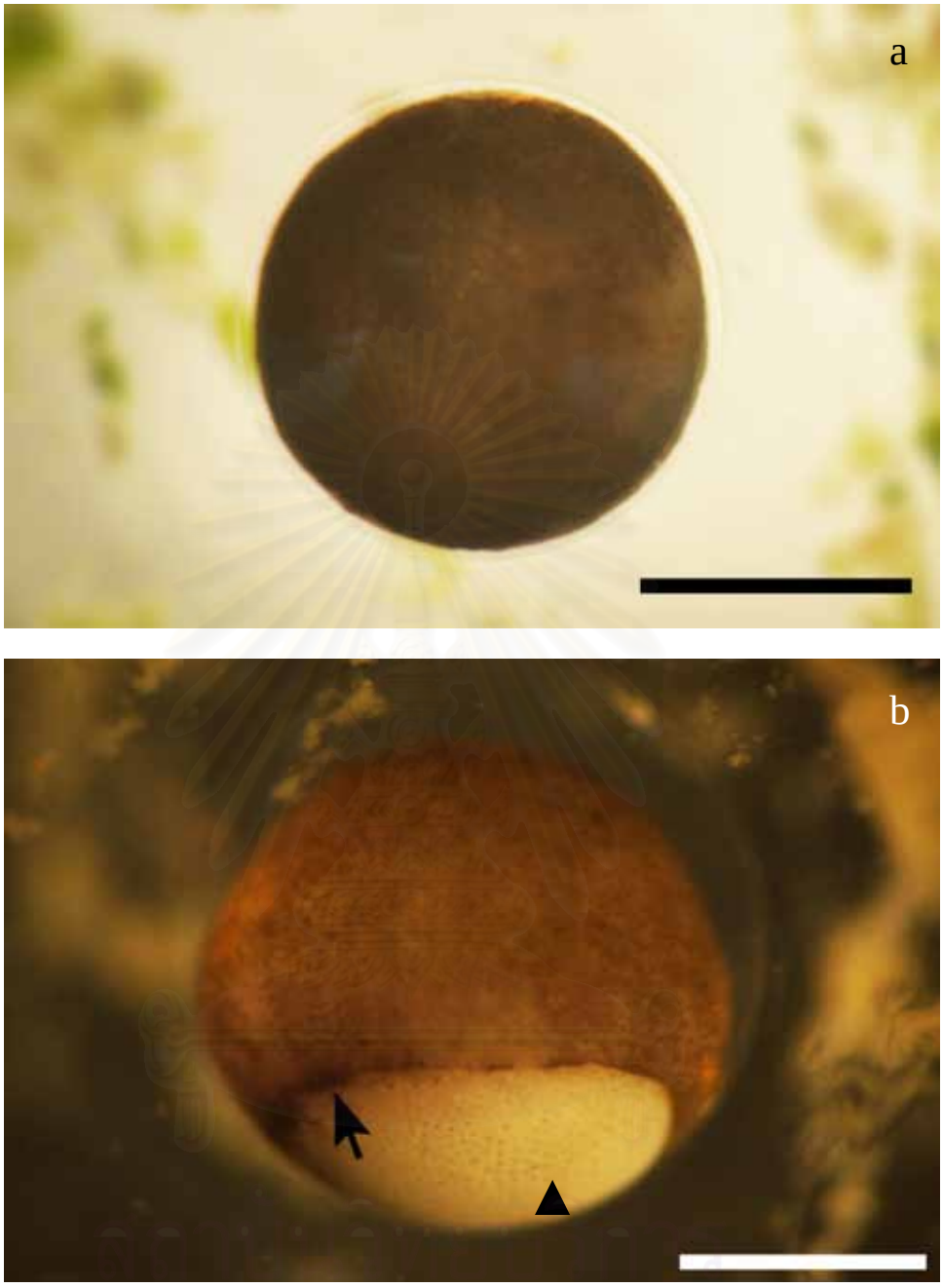


Figure 3-6: Photograph from (a) the animal pole view and (b) the vegetal pole view of *H. rugulosus* development (bar = 1 mm).

(a) Age 7 hrs: late blastula. Determined by small size of macromeres as indicated in picture, the entire surface is smooth. (Nieuwkoop and Faber stage 9, Gosner stage 9)

(b) Age 9 hrs: first appearance of the dorsal lip of blastopore (arrow), beginning gastrulation. (Nieuwkoop and Faber stage 10, Gosner stage 10) (arrowhead-yolk plug)

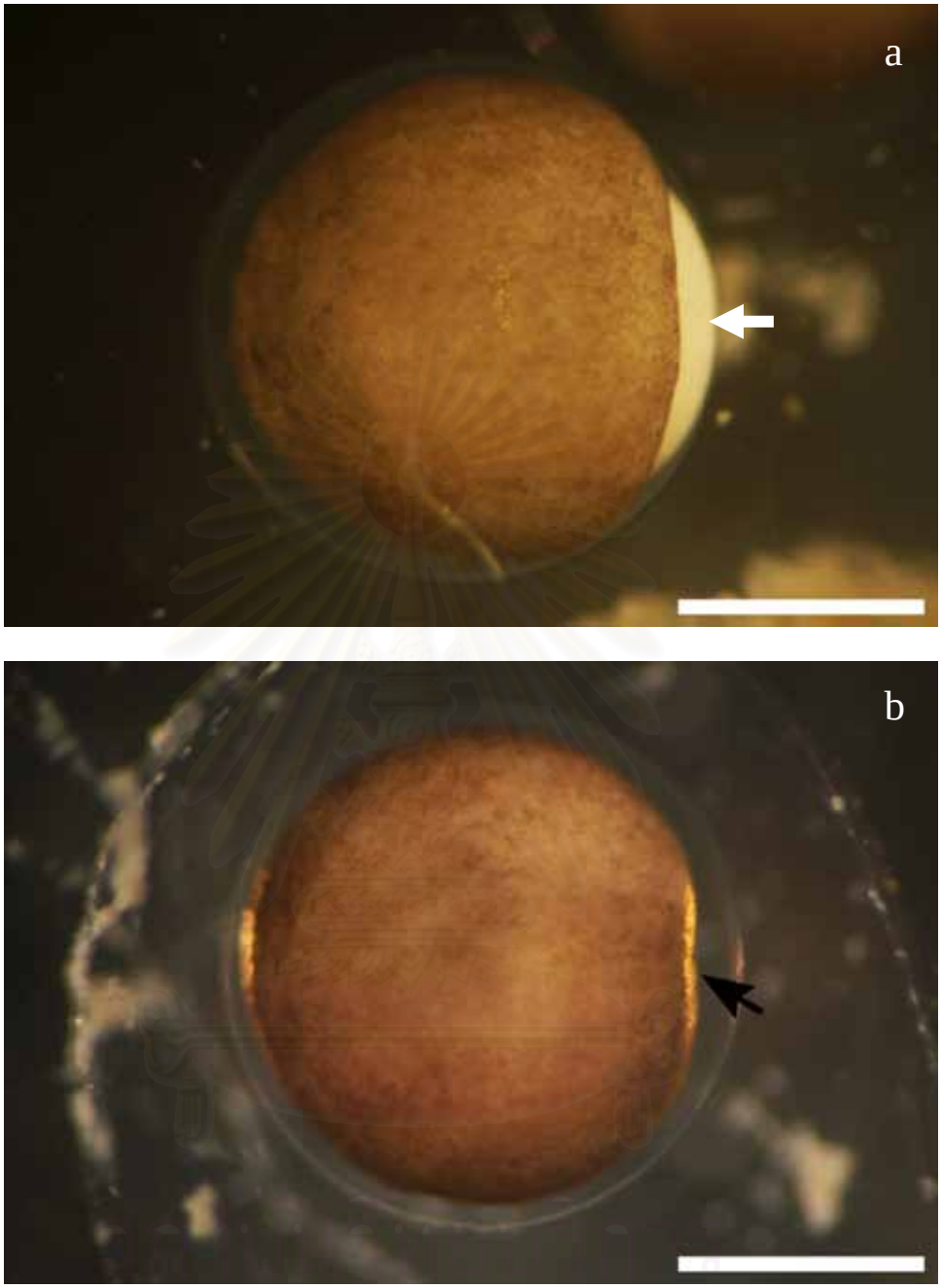


Figure 3-7: Photograph from the dorsal view of *H. rugulosus* development (bar = 1 mm).

(a) Age 11 hrs: mid-gastrula. (Nieuwkoop and Faber stage 10½ , Gosner stage 11)

(b) Age 14 hrs: late gastrula. Blastoporal lip invaginating around circular yolk plug (arrow). (Nieuwkoop and Faber stage 12, Gosner stage 12)

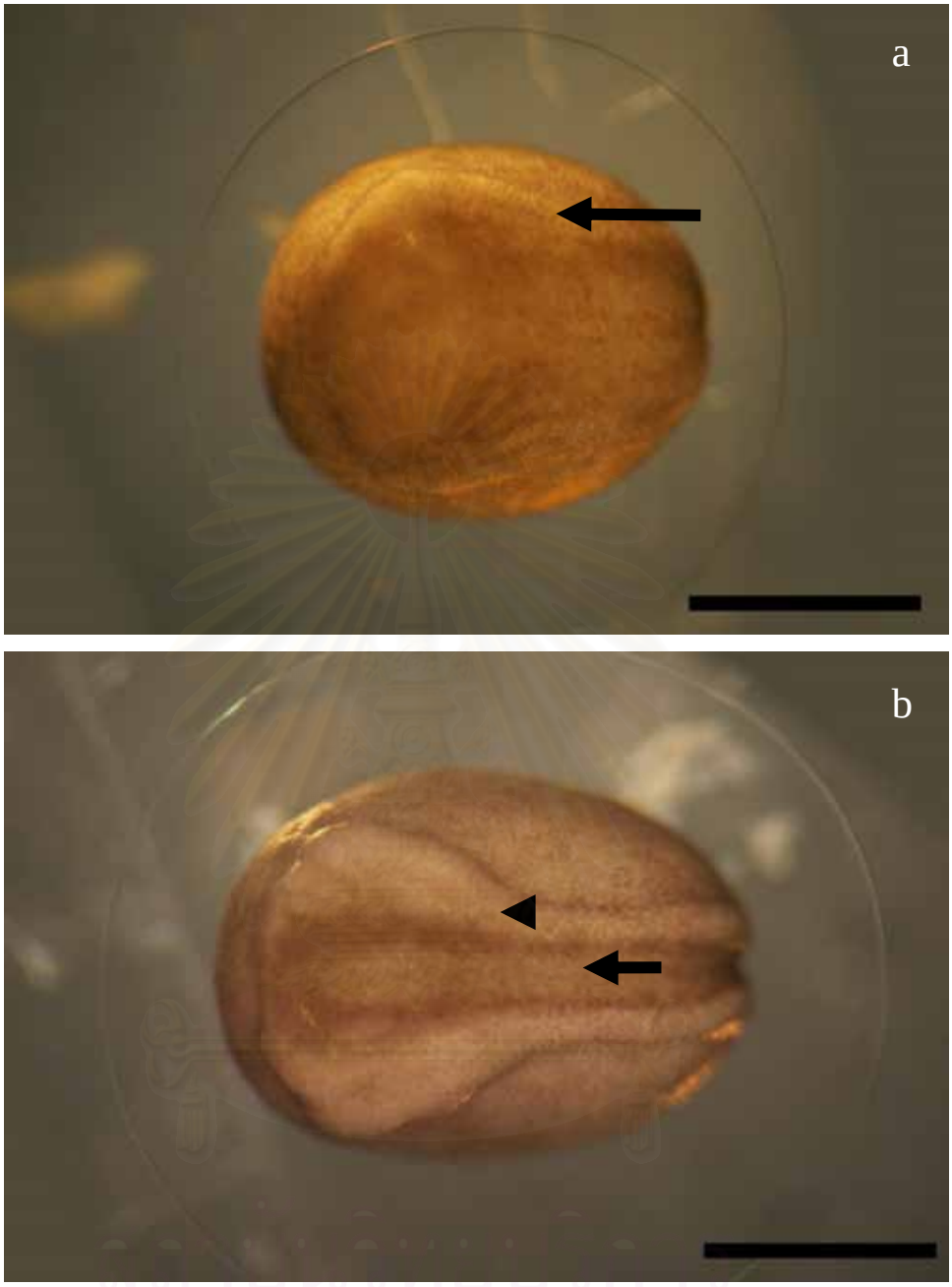


Figure 3-8: Photograph from the dorsal view of *H. rugulosus* development (bar = 1 mm).

(a) Age 16 hrs: neural plate (arrow). (Nieuwkoop and Faber stage 13, Gosner stage 13)

(b) Age 18 hrs: neural folds (arrow) and neural groove (arrowhead). (Nieuwkoop and Faber stage 13, Gosner stage 13)

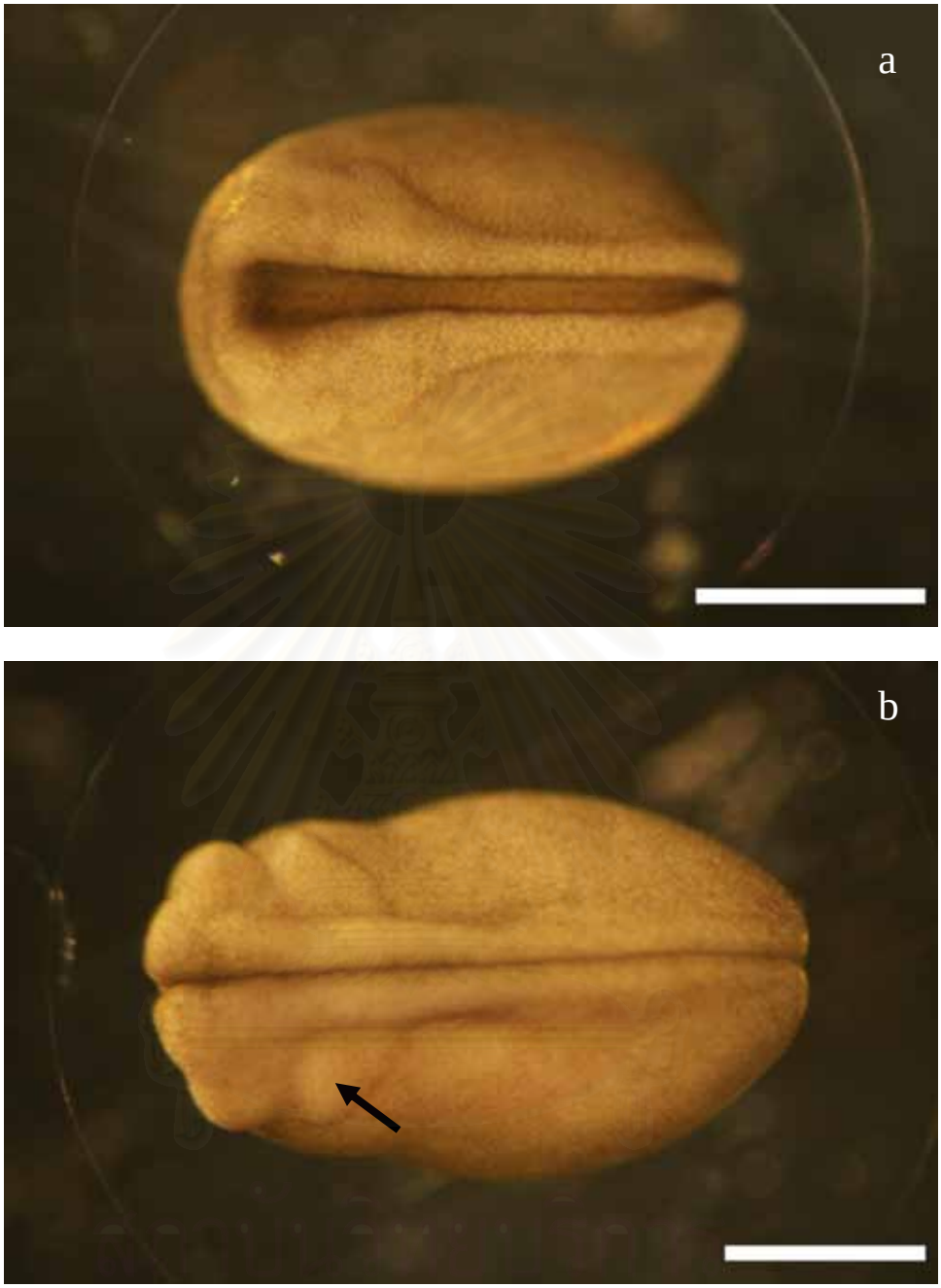


Figure 3-9: Photograph from the dorsal view of *H. rugulosus* development (bar = 1 mm).

(a) Age 20 hrs: mid neural folds stage (Nieuwkoop and Faber stage 15)

(b) Age 20 hrs: neural tube stage. Gill plate can be distinct (arrow). Total length is about 2-3 mm. (Nieuwkoop and Faber stage 17, Gosner stage 15)

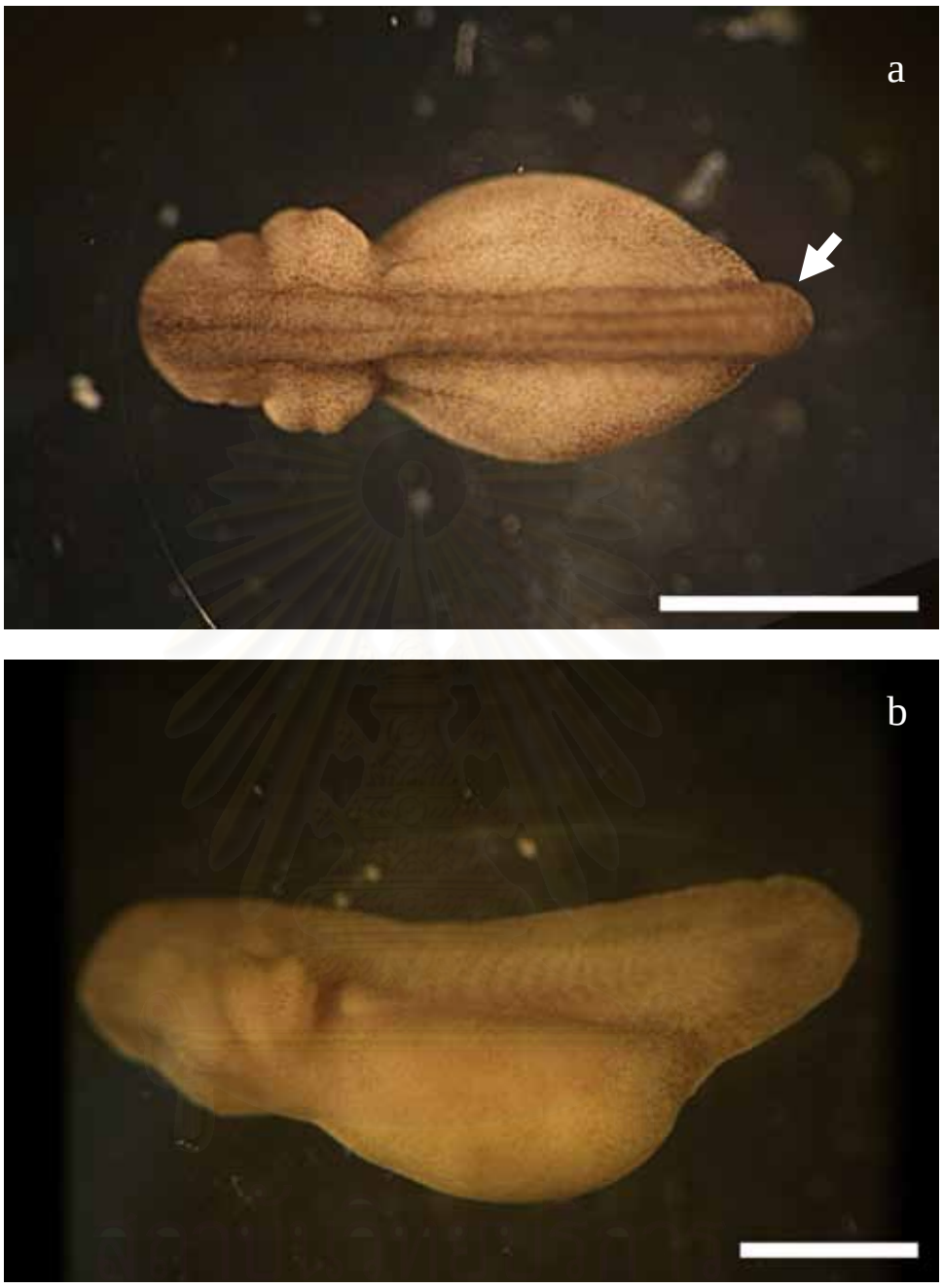


Figure 3-10: Photograph from (a) the dorsal view and (b) lateral view of *H. rugulosus* development (bar = 1 mm).

(a) Age 1 day and 2 hrs: tail bud distinct (arrow). Total length is about 2-3 mm. (Nieuwkoop and Faber stage 23, Gosner stage 17)

(b) Age 1 day and 9 hrs: beginning of muscular response to mechanical stimulation. Tail length is one fourth or one fifth of the length of body plus head. Total length is about 4-5 mm. (Nieuwkoop and Faber stage 26, Gosner stage 18)

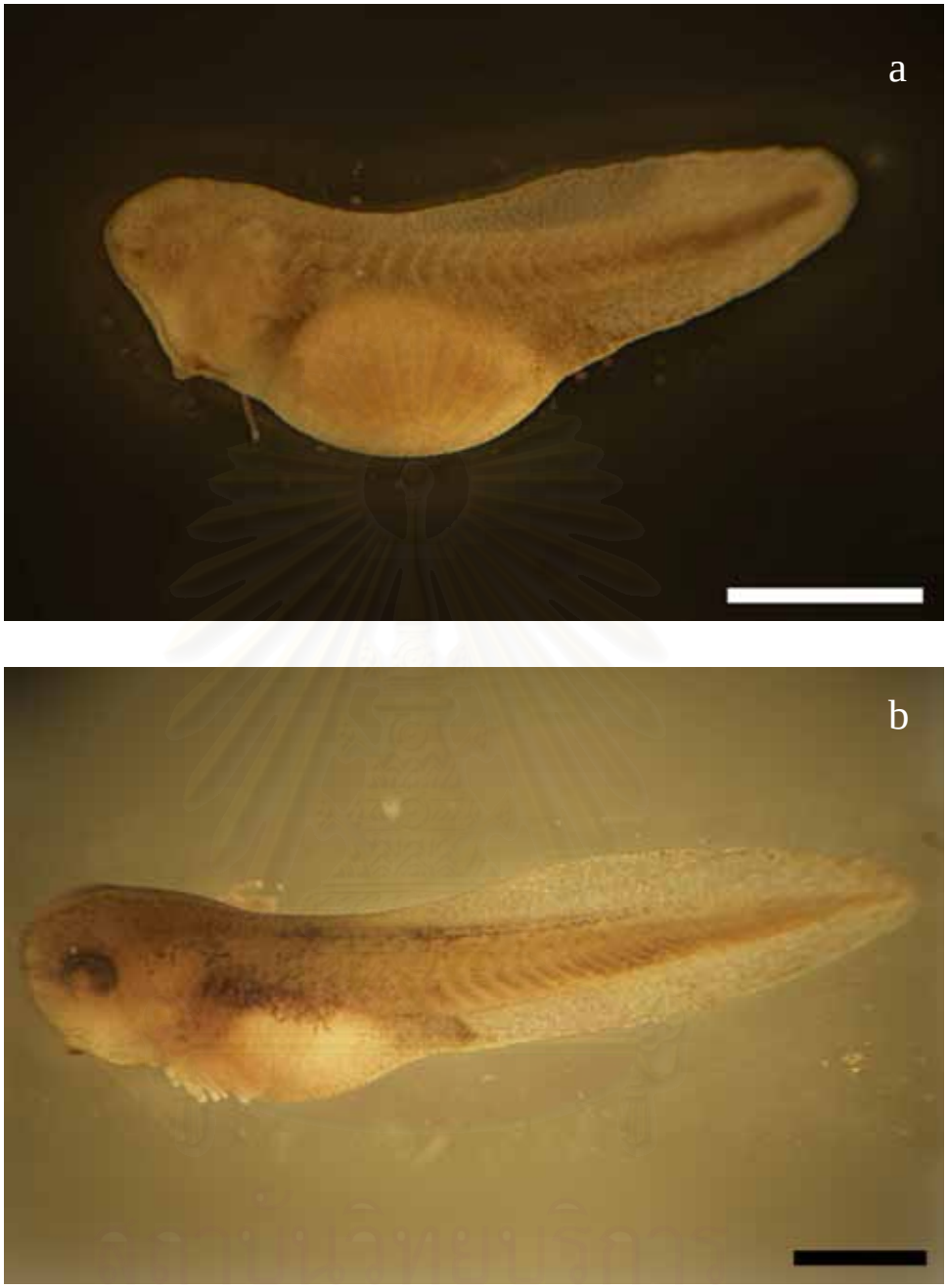


Figure 3-11: Photograph from (a) the dorsal view and (b) lateral view of *H. rugulosus* development (bar = 1 mm).

(a) Age 1 day and 19 hrs: Heart beating and capillary circulation in first external gill. Tail length one-half length of body plus head. Embryo could hatch if it was shaken Total length is about 5-7 mm. (Nieuwkoop and Faber stage 32, Gosner stage 20)

(b) Age 1 day and 22 hrs: mouth open, cornea become transparent, embryo begins to hatch, sucker undergoing involution. Total length is about 5-7 mm. (Nieuwkoop and Faber stage 35, Gosner stage 21)

Figure 3-12: Photograph of limb bud development stage of *H. rugulosus*.

(a) Age 4 days: the operculum (arrow) completely developed. Total length is about 10 mm. (Nieuwkoop and Faber stage 46, Gosner stage 25)

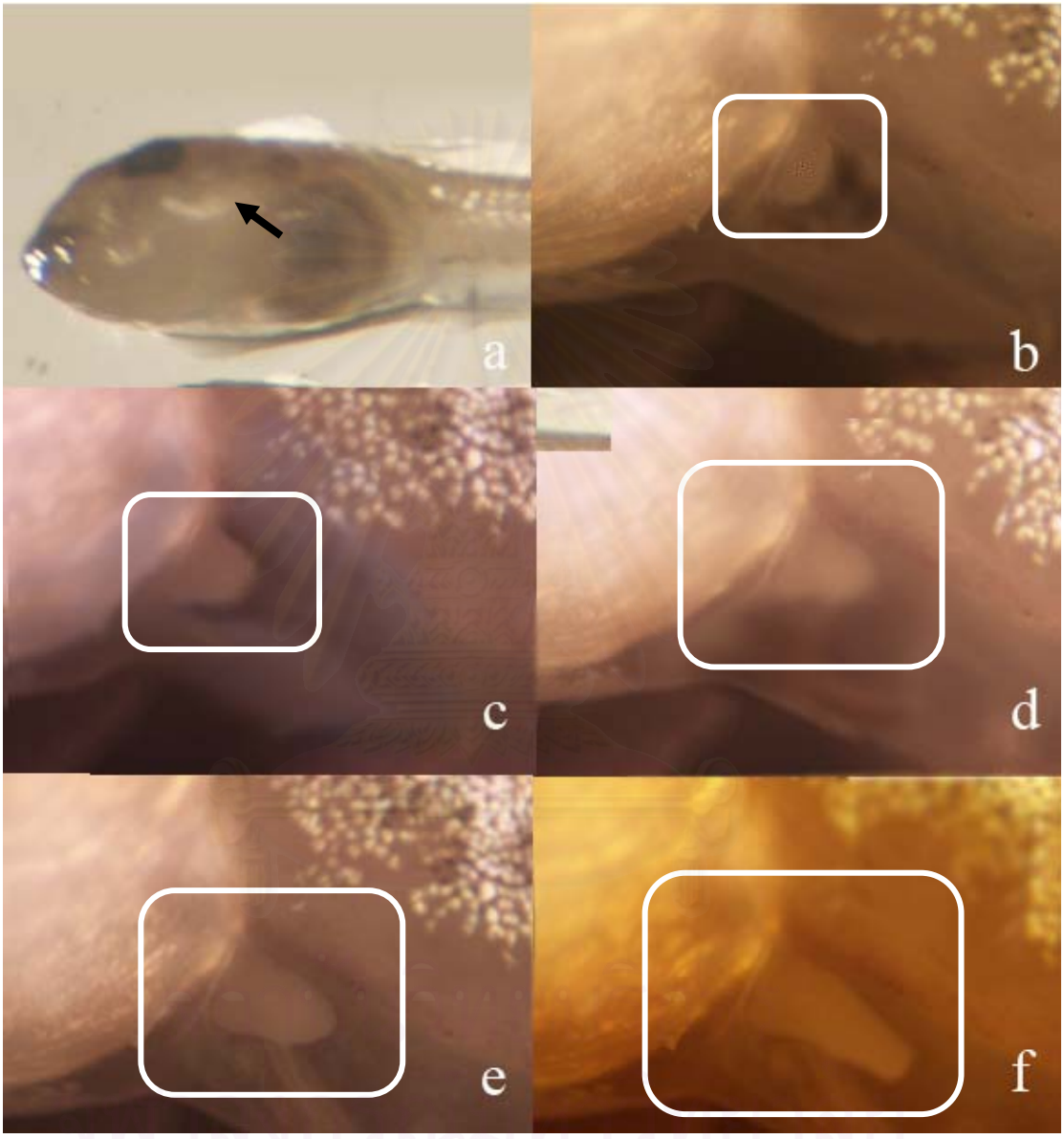
(b) Age 6 days: beginning of hide leg bud development. The height of the limb bud elevation (length of the limb bud) is less than half of its diameter. Total length is about 10-12 mm. (Nieuwkoop and Faber stage 48, Gosner stage 26)

(c) Age 8 days: the length of the limb bud is equal half of its diameter. Total length is about 16-20 mm. (Nieuwkoop and Faber stage 49, Gosner stage 27)

(d) Age 10 days: the length of the limb bud is equal to its diameter. Total length is about 20-22 mm. (Nieuwkoop and Faber, Gosner stage 28)

(e) Age 12 days: the length of the limb is equal to one and one-half times its diameter. Total length is about 20-21 mm. (Gosner stage 29)

(f) Age 15 days: the length of the limb bud is twice its diameter. The distal half of the bud is ventrally bent. Total length is about 20-23 mm. (Gosner stage 30)


a
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c
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e
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Figure 3-13: Photograph of foot paddle stage *H. rugulosus* development.

(a) Age 15 days: the distal end of the limb bud is flattened to form the foot paddle. Total length is about 21-25 mm. (Gosner stage 31)

(b) Age 16 days: the four and fifth toe prominences are separated by a slight indentation of the margin of the foot paddle (arrow head). Total length is about 23-26 mm. (Gosner stage 32)

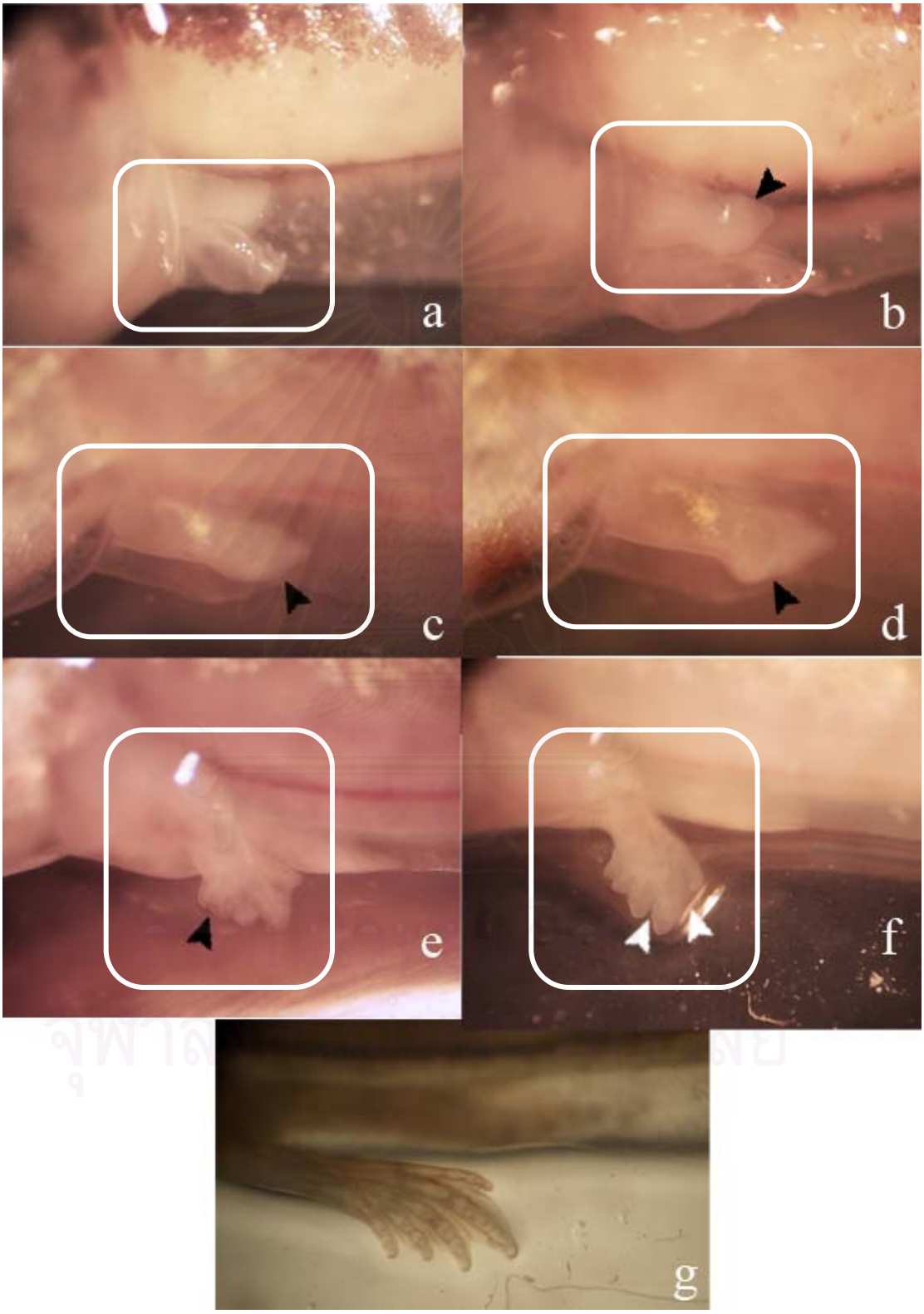
(c) Age 17 days: the margin of the foot paddle is indented between toes 5-4 and 4-3 (arrow head). Total length is about 25-29 mm. (Gosner stage 33)

(d) Age 19 days: the margin of the foot paddle is indented between toes 3-4, 4-3, and 3-2 (arrow head). Total length is about 30-35 mm. (Gosner stage 34)

(e) Age 20 days: the margin of toe foot paddle is indented between all five toes. The margin of the fifth toe web is directed toward the tip of the third toe (arrow head). Total length is about 32-36 mm. (Gosner stage 35)

(f) Age 22 days: the margin of the fifth toe web is directed toward the tip of the second toe (arrow head). Total length is about 35-37 mm. (Gosner stage 36)

(g) Age 25 days: the margin of the fifth toe web is directed toward the tip of the first toe. Total length is about 35-42 mm. (Gosner stage 37)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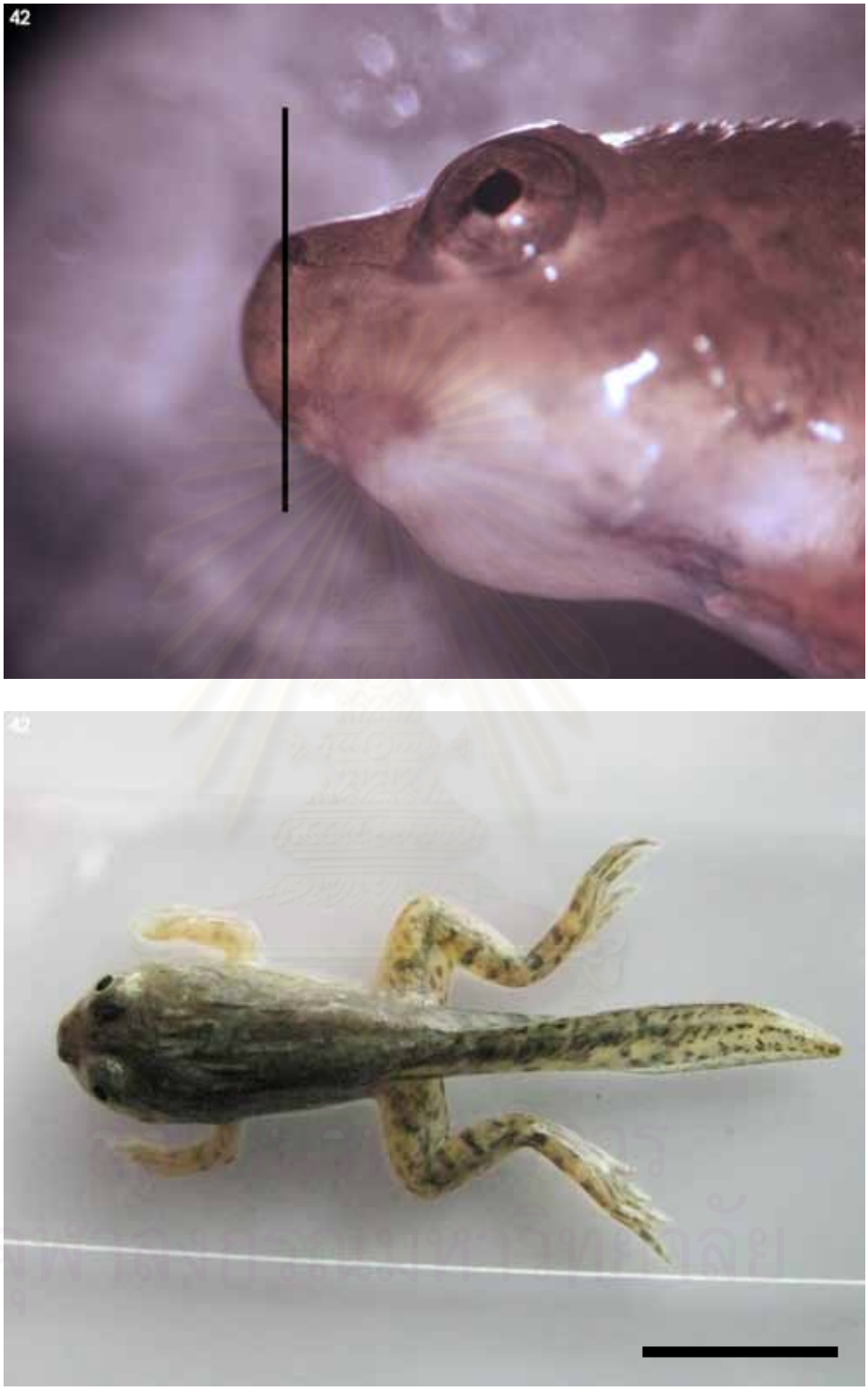

Figure 3-14: Photographs of metamorphosis stages of *H. rugulosus* development at age 28-30 day, indicated by the emergence of forelimb and the angle of mouth is in line with nostril (Nieuwkoop and Faber stage 59, Gosner stage 42) (bar=1 cm).

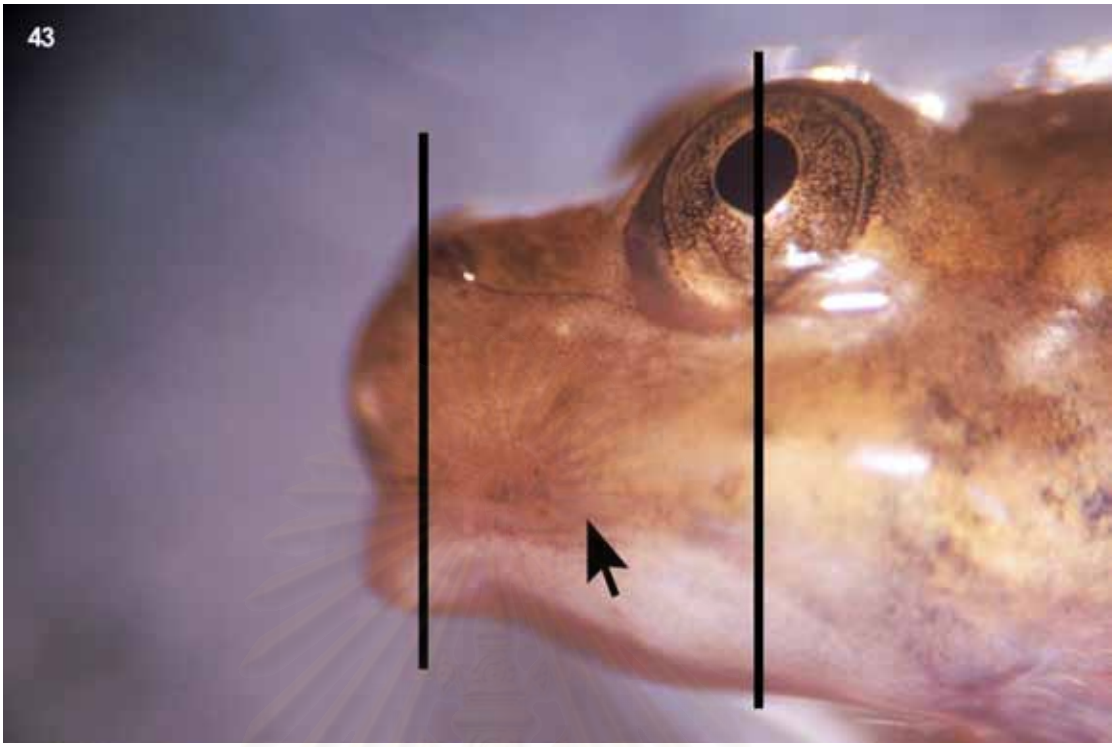

Figure 3-15: Photographs of metamorphosis stages *H. rugulosus* development at age 30-35 days. The angle of the month has reached a point midway between the nostril and the anterior margin of the eye. (Nieuwkoop and Faber stage 61, Gosner stage 43) (bar=1 cm).

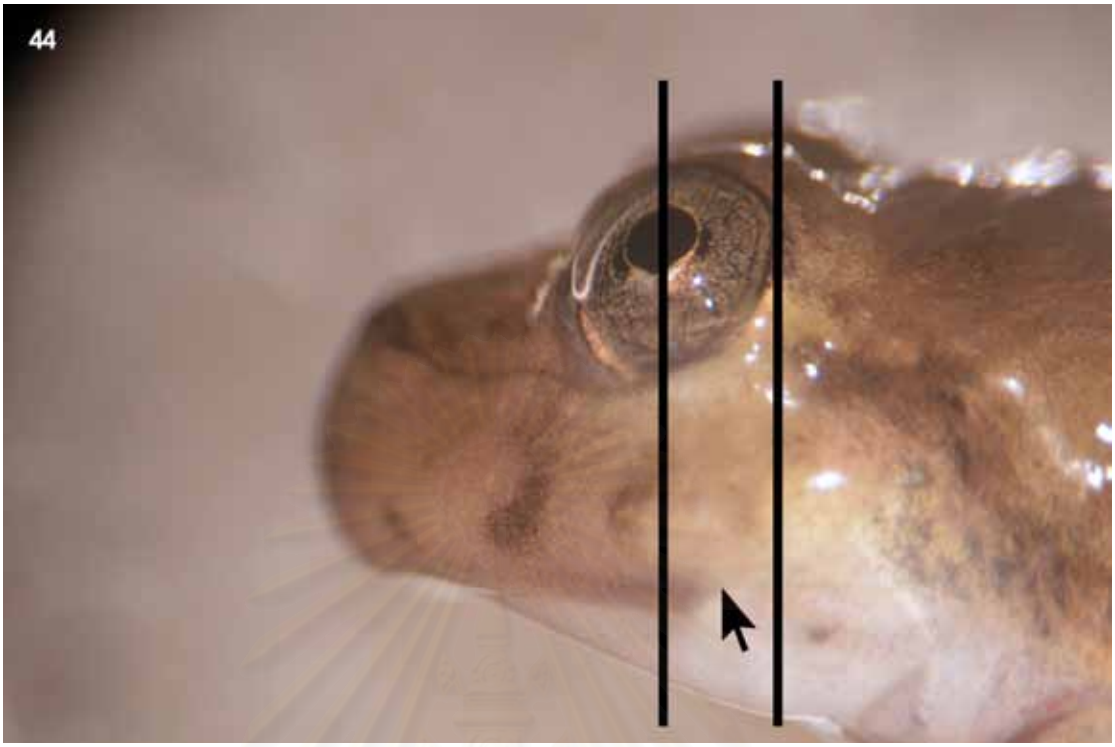

Figure 3-16: Photographs of metamorphosis stages *H. rugulosus* development at age 35-37 days: the angle of the mouth has reached the level of the middle of the eye. (Nieuwkoop and Faber stage 62, Gosner stage 44) (bar=1 cm)

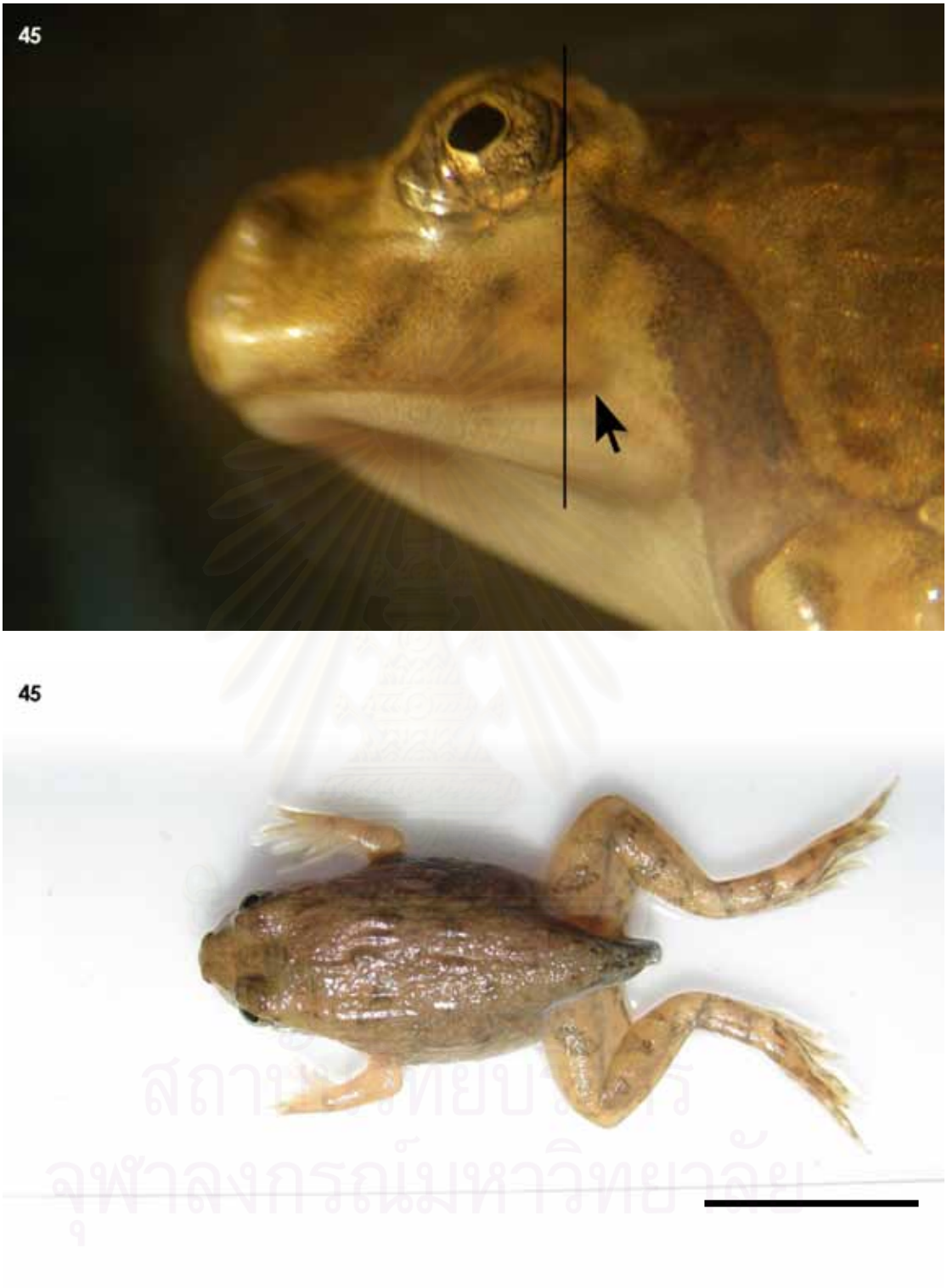

Figure 3-17: Photographs of metamorphosis stages *H. rugulosus* development at age 37-42 days: the angle of the mouth has reached the level of the posterior margin of the eyeball. (Nieuwkoop and Faber stage 65, Gosner stage 45) (bar=1 cm)

Figure 3-18: Photographs of *H. rugulosus* development at the age 45-50 days. The tail is completely gone. The frog reaches the end of metamorphosis. (Nieuwkoop and Faber stage 66, Gosner stage 46) (bar=1 cm)

Discussion

The *H. rugulosus* embryos developed quite satisfactorily in the laboratory and within the 45-50 days the frogs reached the end of metamorphosis at 25 ºC under laboratory conditions. Firstly, the Nieukoop and Faber stages have been used to determine the development of *H. rugulosus* because these are required for the FETAX and developmental biology experiments. The results showed that the *H. rugulosus* embryos can be identified using Nieuwkoop and Faber (1956) stage 1 to 46 of development within 96 hours. Thus, *H. rugulosus* embryos could be considered to be an alternative anuran model for FETAX study in term of development time.

The embryonic development patterns of *H. rugulosus* are also quite similar to others species of anuran (Shumway, 1940; Taylor and Kollros, 1946 and Gosner, 1960). Although the time of development varies regarding temperature and species, those embryonic development stages of *H. rugulosus* could be defined by using the stage of Nieuwkoop and Faber (1956) or Gosner (1960) or Shumway (1940) with few differences. This study could not observe the time after fertilization to the appearance of the grey crescent because this study could not conduct *in vitro* fertilization, however, the time after the spawning to the first cleavage was observed.

The post-embryonic period of *H. rugulosus* was defined using the major indications by Taylor and Kollros (1946), and Gosner stages. Since the Nieuwkoop and Faber stages are specific to *X. laevis* which means some evidence could not be applied to this study. For example, the emergence of forelimbs of *X. laevis* was found coincided with the development of foot paddle stage of hindlimb. In contrast, *H. rugulosus* forelimb emerged after foot paddles development which similar to Taylor and Kollros, and Gosner stages.

In conclusion, *H. rugulosus* could be considered as one of the good alternative anuran models for studying embryology and developmental biology in South East Asia region instead of *X. laevis*. However, further studies such as the in depth study of histology or physiology of embryos and larvae should be undertaken.

CHAPTER IV

EFFECTS OF ATRAZINE ON THE EARLY DEVELOPMENT OF THE RICE FIELD FROG *Hoplobatrachus rugulosus* (Wiegmann, 1834)

Introduction

Embryonic and early life stages of development are sensitive to toxic substances or environmental contaminants; as a result, embryo mortality, malformation, and growth inhibition can often occur at concentrations lower than those affecting adults (ASTM, 2004). Thus, for anuran eggs laid and developing in such water, the contaminants would be directly affecting them.

As for the increasing chemical contamination of the environment which may affect many organisms including humans, animal models and scientific protocols were used to evaluate the effect of chemical reagents. The Frog Embryo Teratogenesis Assay for *Xenopus* (FETAX) is a protocol developed by Dumont *et al*. in 1983 which was standardized and certified by the American Society of Testing and Materials (Fort *et al.*, 2004). The protocol is used to identify developmental toxicants and is performed by exposing the African clawed frog (*X. laevis*) embryo to a direct concentration of toxicants for 96 hours then observing the mortality and abnormality which occurs. This protocol is considered to be inexpensive, fast, and a reliable assay that is used as a screening model for testing the teratogenecity of chemicals (Morgan *et al.*, 1996). FETAX data may also be inferred for other species including mammals (ASTM, 2004).

There are few studies on the effects of atrazine on amphibian using the FETAX technique. Morgan *et al* (1996) examined the effects of atrazine dissolved in

both FETAX buffer and natural water, demonstrating that EC_{50} was 33 ppm and LC_{50} was 100 ppm in FETAX buffer. However, the result from atrazine dissolved in natural water caused the EC_{50} to be less than 8 ppm while the LC_{50} was 126 ppm. The author suggests that the higher toxicity of atrazine in natural water may result from the enhanced effects of some compounds in water or atrazine which was already present in the sample (Morgan *et al*., 1996). Fort *et al.* (2004) reported the effects of atrazine in *X. laevis* that EC_{50} was 4.3 ppm, LC_{50} was 25.1 ppm. Moreover, some characteristic malformations were reported such as, notochord lesions, tail flexures, abnormal mouth development at 5 ppm in *X. laevis*. At 1.0 ppm, *X. tropicalis* developed abnormal mouth and craniofacial features (Fort *et al*, 2004).

The present study was carried out using *H. rugulosus* to test whether atrazine at ecologically relevant concentrations would have effects on embryonic development following the FETAX protocol. However, certain steps in the procedures were modified as suggested by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC).

Materials and methods

All test substances used in this study were purchased from Ajex Finechem Pty. Ltd., (New South Wales, Australia) except atrazine and L-cystein which were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

FETAX solution was used as the solvent in this experiment, containing: 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$ per liter of double distilled water (pH = 7.60) (ASTM, 2004).

Adult rearing and breeding procedures was described in the previous chapter. After eggs were laid, the adults were removed. Success rates of fertilized eggs were checked by observing the presence of a fertilization membrane with the dissecting microscope (Olympus® SZ230). Only batches of eggs with a high percentage of fertilization (more than 90%) were used for further study.

Mid blastula embryos (stage 8 of Nieuwkoop and Faber, 1965) were selected and harvested with a Pasteur pipette and placed into FETAX solution. The embryo's jelly coat was removed by gentle swirling in 2 % L-cystein w/v (pH 8.0) in FETAX solution while being observed under dissecting microscope (Figure 3-1). After removing the jelly coat, the L-cystein solution was drained and the eggs rinsed with FETAX solution 4-5 times. The de-jellied eggs were checked again for normality before being randomly transferred to 10 mm Petri dishes containing the test solution, with 30 animals per dish. The experiment was conducted at 25 ± 2^{o} C, 12h/12h light dark cycle.

The nominal concentrations of atrazine in this study were; 0.001, 0.01, 0.1, 1, 10, 100, 1000 ppb with 3 replicates. The atrazine was pre-dissolved in absolute ethanol (Hayes, 2002). The final concentration of ethanol was less than 0.001% v/v,

however, the negative control was provide with 0.001% v/v ethanol. Then, atrazine was dissolved in FETAX solution. Each Petri dish contained 10 ml of testing solution which was renewed every 24hr. The tests end when larvae in the control groups reached Nieuwkoop and Faber stage 46, fully develop of operculum stage, or 96 hours post fertilization

Data collection and statistical analysis

For each FETAX test, frequencies of mortality and malformation were determined. The abnormality was define follow the score sheet of malformation (ASTM, 2004; Figure 3-2). Snouth-vent length of surviving larvae was measured through photographed of the larvae (Olympus® E220) through dissecting microscope (Olympus® SZ230) and length were measured with program Image-J studio (public domain software from NIH, USA). All data were statistically tested for normal distribution and homogeneity of variance using SigmaStat® 3.5 for Windows. The parameter which accept the normal distribution pattern were compared by the Analysis of Variance (ANOVA) at $p<0.05$, using SigmaStat® 3.5 for Windows. The parameter which failed the normal distribution pattern were compared by the Kruskal-Wallis Analysis of Variance at $p<0.05$, also using SigmaStat® 3.5 for Windows.

Results

The temperature and pH in this experiment ranged from 24-26 ºC and 6.5-7.0, respectively. After 96 hours test, all of animals in the control groups and all treatments of *H. rugulosus* reached Nieuwkoop and Faber stage 46 of development

The mortality ranged from 10.00-21.11% (Table 4-1). The solution and ethanol controls mortality were 16.67±5.78% and 13.33±6.67%, respectively. The statistically analysis, however, showed that there were no statistical differences between the control solution and the ethanol control ($p<0.05$). In treatment groups, the highest mortality was found in 1,000 ppb of atrazine (21.11±15.40%) and the lowest was 1 ppb (10.00±5.77%). There was no statistically difference among all treatments of atrazine compared with both control groups (ANOVA, $p<0.05$).

The results of the head-tail lengths of the control and all treatments groups ranged from 8.90-11.59 mm (Table 4-1). The solution control and ethanol control snout-vent length also showed no statistical differences ($p<0.05$). The highest snout-vent length value found in 1,000 ppb atrazine groups (11.59±0.07 mm) and lowest in 0.01 ppb atrazine groups (8.90±0.40 mm); however, there were also no statistical differences among all groups (Kruskal-Wallis One Way analysis of Varience, $p<0.05$).

The abnormalities found were tail flexures and abdominal edemas (figure 3-1) in all treatment groups except 0.01 ppb. There were no abnormalities observed in both control groups. In treatment group, the abnormalities were highest in 0.1 ppb atrazine (13.82±3.88%) and lowest in 0.01 ppb atrazine (0%). Nevertheless, the statistical analysis also reported no significant differences among all groups (Kruskal-Wallis One Way analysis of Varience, $p<0.05$).

Table 4-1: Result of the average mortality, head-tail length and malformations of atrazine on *H. rugulosus*.

Concentration (ppb)	Mortality (percent±SEM)	Head-tail length (mm±SEM)	Malformation (percent±SEM)		
			Tail flexure (%)	Abdominal edema (%)	Total (%±SEM)
control	16.67 ± 3.33	10.63 ± 0.25	0	0	0
control ethanol	13.33 ± 3.85	10.88 ± 0.69	0	0	0
0.001	12.22 ± 2.22	10.23 ± 0.19	6.33	2.53	8.74 ± 6.84
0.01	15.56 ± 4.01	8.90 ± 0.40	0	0	0
0.1	18.89 ± 11.6	10.36 ± 0.08	12.33	2.74	13.83 ± 3.88
1	10.00 ± 3.33	10.06 ± 0.40	9.88	1.23	11.27 ± 3.99
10	11.11 ± 4.84	10.31 ± 0.34	3.75	0	3.69 ± 2.01
100	18.89 ± 5.88	9.90 ± 0.40	6.85	0	7.08 ± 1.74
1000	21.11 ± 8.89	11.59 ± 0.08	5.63	2.82	9.09 ± 5.00

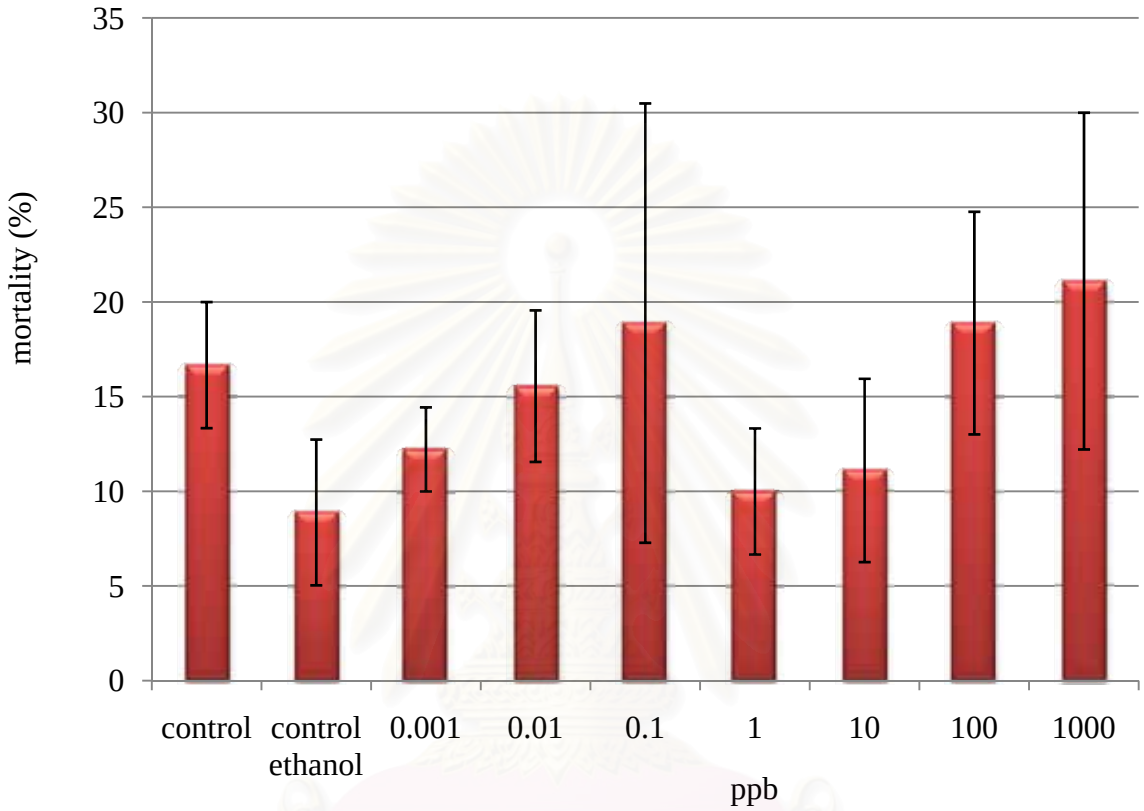

Figure 4-1: Average mortality during early development of *H. rugulosus*treated with atrazine.

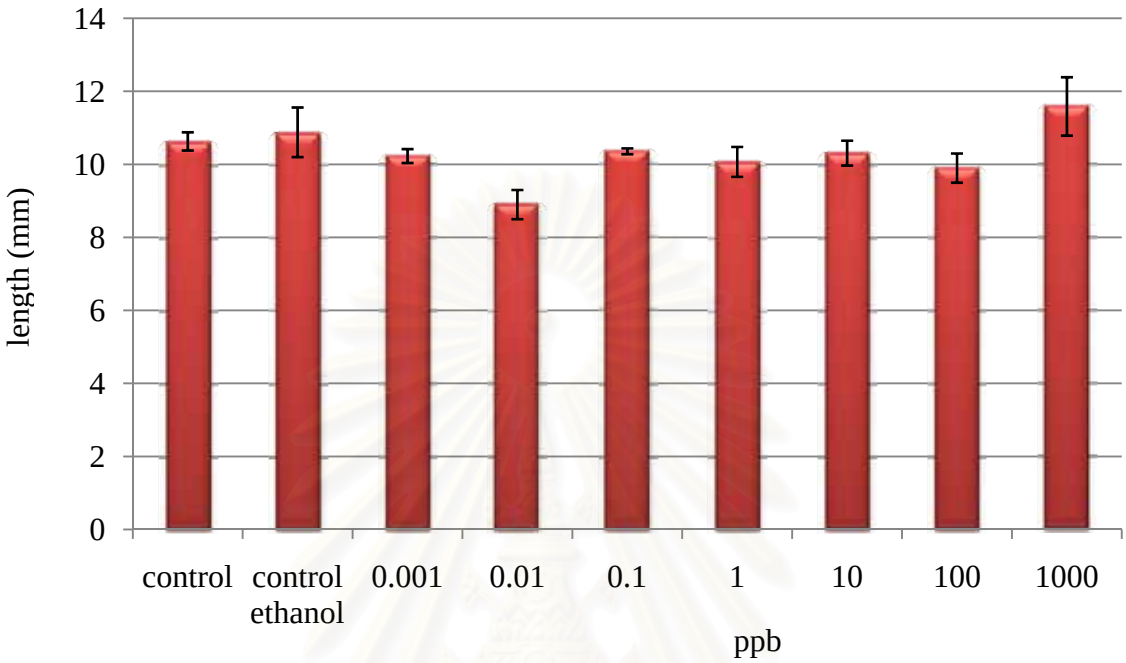

Figure 4-2: Effect of atrazine on average head tail length of early hatched larvae of *H. rugulosus*.

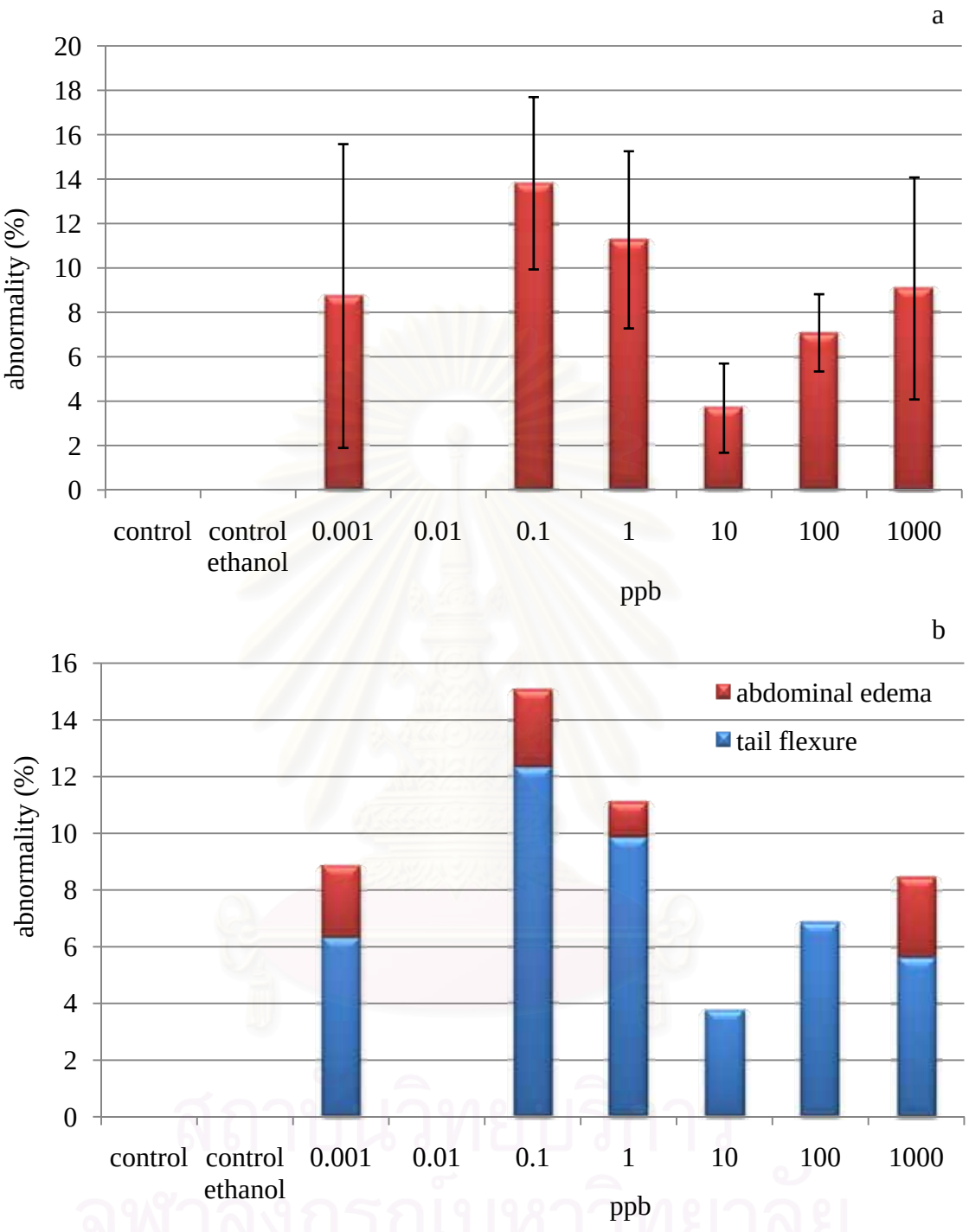

Figure 4-3: Effect of atrazine on (a) total average and (b) categorized by abnormalities of embryonic abnormalities during early development of *H. rugulosus*.

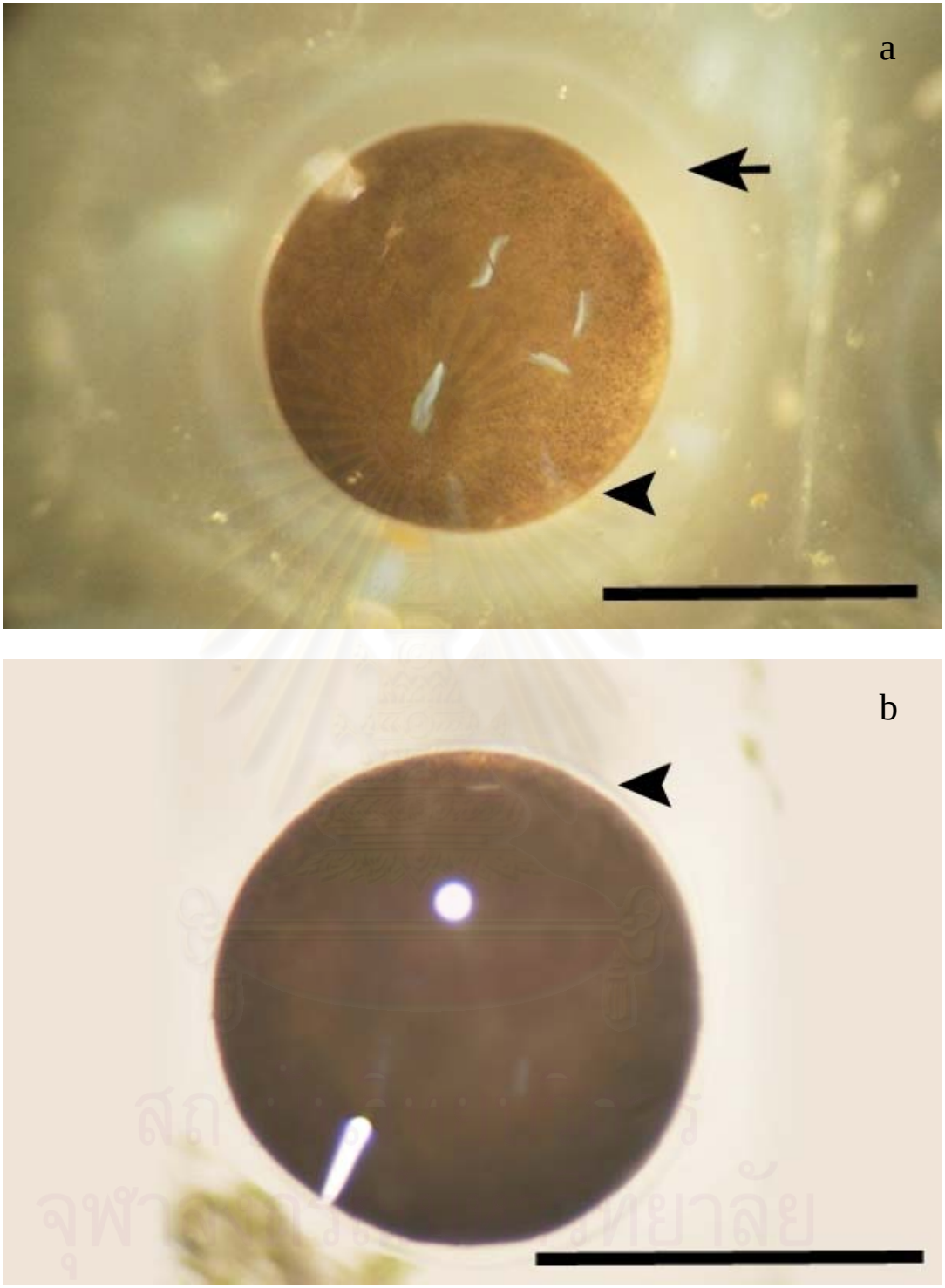

Figure 4-4: Development at late cleavage stage (Nieuwkoop and Faber stage 8) of *H. rugulosus* (a) with jelly coat (b) without jelly coat

(arrow=jelly coat, arrow head=fertilization membrane, bar = 1 mm)

Figure 4-5: A 100 mm Petri dish containing 30 *H. rugulosus* larvae at 96 hours post fertilization (Nieuwkoop and Faber stage 46)

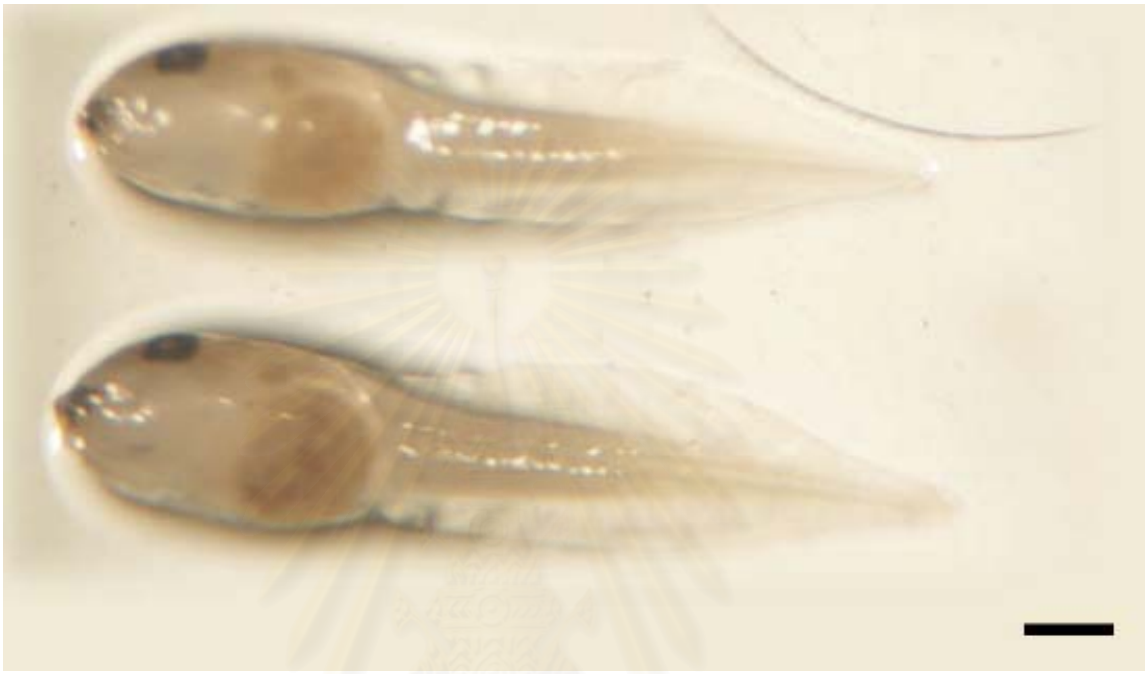

Figure 4-6: Nieuwkoop and Faber developmental stage 46 of normal *H. rugulosus* larvae (bar = 1 mm)

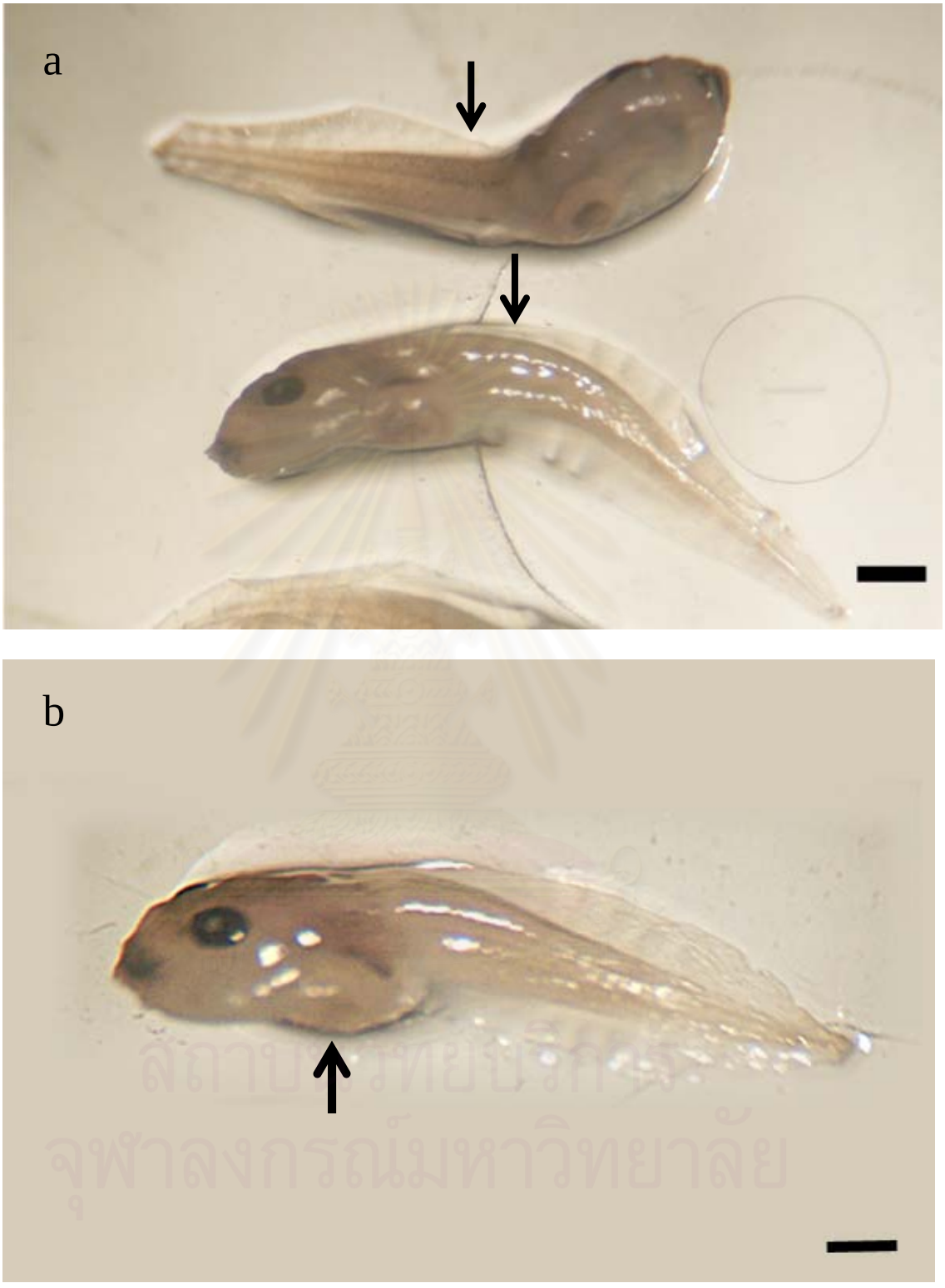

Figure 4-7: Malformations observed at Nieuwkoop and Faber stage 46 (a) tail bent and (b) abdominal edema (arrow) (bar = 1 mm)

Discussion

This study using a modified FETAX method to evaluate whether atrazine at ecologically relevant concentrations would have any effects on local amphibian development by using *H. rugulosus* embryos instead of *X. leavis* embryos. The results demonstrated that the nominal concentrations of atrazine in this study showed no acute lethal response to *H. rugulosus* embryos and early larvae. Regarding the fact that there were no statistical differences in the mortality rate in all groups; the nominal concentrations of atrazine used in this study also showed no effect on the mortality of the embryos. The recent reports of lethal concentrations of atrazine in frogs using FETAX protocol were much higher than this study. The study from Morgan *et al*. (1996) reported the LC_{50} was 100 ppm and from Fort *et al*. (2004) LC_{50} was 25.1 ppm in *X. laevis* and in *X. tropicalis* LC_{50} was 20.9 ppm. Furthermore, experiments which did not used FETAX protocol also report toxic concentrations higher than this study, for example, Allran and Karasov (2001) reported LC_{50} for all 3 species of frog (Leopard frog *Rana pipiens*, Wood frog *Rana sylvatica*, and American toad *Bufo americanus*) were more than 20,000 ppb, and from Birge *et al*., (1983) LC_{50} in American toad were more than 4,800 ppb. Regarding the previous studies and our study, the atrazine at the nominal concentrations (0.001 to 1,000 ppb) which found in aquatic environment are not causing the acute effect to *H. rugulosus* and probably to amphibians in the wild.

The head-tail length values which referred to the development of embryo and larvae also showed no statistical differences in all groups, which could suggest that the nominal concentration of atrazine could not directly effect the early development of *H. rugulosus*.

The percent malformations observed also showed no statistical differences among the groups, however, the malformations were only found in the treated groups and appeared not to be in the dose-response pattern. The abnormalities observed were tail flexure and abdominal edema. These malformations were previously reported in *X. laevis* > 1.1 ppm (Morgan *et al*., 1996); > 5 ppm (Fort *et al*., 2004); >25 ppm (Lenkowksi *et al*., 2008). Compared with this study, those malformations were significantly found in the higher atrazine concentration than this study.

The larvae developmental stages just before metamorphosis are important (EPA 2006). In this stage (Nieuwkoop and Faber 37-46), tadpoles are no longer able to rely on the energy stores of the embryonic yolk. Many organs undergo rapid morphogenesis to prepare for the onset of feeding. Many factors contribute to this intricate process of organ morphogenesis that was observed to be disrupted after atrazine exposure (Lenkowksi *et al*. 2008).

Previously, the atrazine test using FETAX reported abnormalities at higher concentration of atrazine than this study. Morgan *et al.* (1996) reported that EC_{50} was 33.0 ppm. The abnormalities also found were tail flexure or gut edema. Fort *et al*. (2004) also reported that atrazine caused *X. laevis* notochord lesions, tail flexures, craniofacial defects, microphthalmia, lens and pigmented retina malformations and abnormal mouth developments at 5.0 ppm, moreover, atrazine at 25.0 ppm also induced micro-cephaly, visceral hemorrhages and cardiovascular malformations. In *X. tropicalis*, 1.0 ppm atrazine induced abnormal mouth and craniofacial developments and at 10.0 ppm induced notochord lesions, tail flexures, microphthalmia, ruptures of pigmented retina.

According to the none-significant different among all groups though using different criteria; therefore, it cannot conclude that the nominal concentrations of atrazine used in this experiment effect the embryonic and early larvae development of *H. rugulosus*.

CHAPTER V

EFFECTS OF ATRAZINE ON THE GONAD DEVELOPMENT OF THE RICE FIELD FROG *Hoplobatrachus rugulosus* (Wiegmann, 1834)

Introduction

Atrazine was detected in natural aquatic systems in many areas in the U.S. and in Thailand through run off or direct applications. Atrazine is generally applied in the rainy season when anuran species congregate to breed. Consequently, anurans could be affected by atrazine contamination. Most concentrations of atrazine detected in the aquatic environment do not cause acute death; however, they could suffer from sub-lethal effects. To evaluate the effects of atrazine on amphibian development, researchers tested a variety of frog species, including the African clawed frog (*Xenopus laevis*), the Leopard frog (*Rana pipiens*), and the Green frog (*R. clamitans*) which addressed a variety of endpoints: time to metamorphosis, growth (body length and weight), gonadal abnormalities, sex ratios, laryngeal dilator muscle area, plasma steroid concentration, and brain/gonad aromatase activity.

Tavera-Mendoza *et al* (2001a, 2001b) reported male and female *X. laevis* exposed to 21 ppb atrazine under static conditions for 48 hrs. In male frogs, the results showed 57% with a significant decrease in total testicular volume. The number of spermatogonial cell nests decreased significantly and the number of nursing cells also declined significantly. Testicular resorption was observed in 70% of the male tadpoles exposed to atrazine relative to controls; failure of full development of the testis (aplasia) was observed in 10% of the testes examined. Histological examination of the pituitary suggested that tissues were actively secreting hormones based on the absence

of chromophores. In female frogs, the frequency of occurrence of primary oogonia was significantly ($p<0.05$) lower in atrazine exposed larvae; however, the frequency of occurrence of secondary oogonia was significantly higher in atrazine-exposed tadpoles. Furthermore, there was no histological evidence that the pituitary was actively secreting hormones. The authors suggested that atrazine may be affecting aromatase activity.

Hayes, *et al*. 2002a. conducted experiments in *Xenopus laevis* exposed under static renewal conditions (complete exposure water change every 72 hrs) to nominal atrazine concentrations ranging from 0.01 to 200 ppb, from 96 hours post-hatch through complete tail resorption. The results showed exposure to atrazine concentrations of more than 0.1 ppb resulted in gonadal abnormalities in 16-20% of the animals. The abnormalities are multiple gonads or hermaphrodites (multiple testes and ovaries in the same animal) whereas these abnormalities were not observed in the controls. Moreover, 80% of the exposed males had below average laryngeal muscle diameters. The authors proposed that one possible mechanism for these phenomena could be that the exposed larvae increased endogenous estrogen concentrations which would be through increased aromatase activity similar to adult males *Xenopus* exposed to atrazine which had significantly reduced plasma testosterone at atrazine 25 ppb. However, the results showed no dose-response relationship to gonadal effects. Hayes *et al.* (2002b) also observed in previous studies of non-native *X. laevis* exposed to atrazine were also exhibited in a native species, *i.e.*, *R. pipiens.* Leopard frog larvae were exposed from 48-hrs post-hatch through complete tail resorption (Nieuwkoop and Faber stage 66) to nominal atrazine concentrations of 0.1 and 25 ppb (0.0036% ethanol) in 10% Holtfreter's solution. The males treated with atrazine at 0.1 and 25 ppb, respectively, suffered from gonadal dysgenesis (under-developed testes with

poorly structured, closed lobules and low to absent germ cells). Furthermore, 29% of the 0.1 ppb and 8% of the 25 ppb animals displayed varying degrees of sexual reversal; testicular lobules of sex-reversed males contained oocytes, and males that metamorphosed later also contained large numbers of oocytes.

Steeger and Tietge (2003) report males frogs *X. laevis* which were exposed to nominal atrazine concentrations of 25 ppb for 26, 43 and 47 days to test whether the pesticide could up-regulate aromatase activity in sexually mature male and female *X. laevis* and to evaluate whether atrazine would decrease plasma testosterone and increase estradiol (consistent with an increase in aromatase activity) in frogs. Of all the atrazine treated periods tested, mean brain aromatase activities from atrazine-exposed males were not statistically different from the reproductive control group. The authors also investigated *X. laevis* larvae exposed to atrazine from 72 hrs after hatching until completion of metamorphosis (Coady *et al*. 2004). In this study, *X. laevis* larvae were exposed to atrazine at nominal concentrations of 0.1, 1.0, 10, and 25 ppb with positive (0.1 ppb 17-β estradiol and 0.1 ppb dihydrotestosterone) and solvent (0.005% ethanol) controls. The authors concluded that atrazine treatment did not effect mortality, time to metamorphosis, sex ratio, gonadal development, aromatase activity or plasma steroid hormone concentrations in a dose-dependent fashion. They also concluded that estradiol (positive control) treatment only appeared to increase plasma estradiol concentrations. Dihydrotestosterone (positive control) increased larynx dilator muscle area in females and neither positive control influenced sex ratios.

Goleman and Carr (2003) exposed 48- to 72-hours post-hatched *X. laevis* larvae to a 78-day exposure at nominal concentrations of 1, 10 and 25 ppb atrazine in FETAX medium, 0.1 ppb 17-β estradiol, 0.1 ppb dihydrotestosterone, and solvent

control (0.0025% ethanol). In this study, atrazine did not effect length, weight, time to metamorphosis or dilator muscle area relative to the controls. However, exposure to 25 ppb atrazine appeared to significantly increase the number of intersex males and animals with discontinuous gonads.

In field studies in the continental USA following a west-east transect from Utah to Iowa, the wild *R. pipiens* from a variety of habitats in areas with reportedly low- and high-atrazine usage were observed by Hayes *et al*. (2003). Field collection of frogs and water samples proceeded eastward, starting in Utah on July 15, 2001 and ending in Iowa on July 28, 2001. At each site, the researchers collected 100 leopard frogs for histological analysis and 100 ml of water for chemical analysis. The researchers identified testicular oocytes in males from seven of the eight collection sites. All sites with atrazine levels exceeding 0.2 ppb had males that displayed sex-reversal similar to those abnormalities induced by atrazine in the laboratory study.

Steeger and Tietge (2003) also report that there were also conducted the effect of atrazine exposure on gonadal abnormalities in *X. laevis* metamorphosis and sub-adults under semi-natural conditions in microcosms with four nominal treatments: 0, 1, 10 and 25 ppb atrazine. The results showed gonadal deformities in 1, 10 and 25 ppb atrazine groups was 1.3, 0.7 and 3.3% of the total frogs examined (150), respectively; control frogs exhibited a 4% incidence of gonadal deformities. The gonadal abnormality identified was only discontinuous testis and no abnormalities were observed in the ovaries except for one ovary that was reduced in size. In addition, male and female frogs not exposed to atrazine were larger, in terms of length and weight than their atrazine-treated groups.

The field survey of adult and juvenile cricket frogs (*Acris crepitans*) to determine whether sexual development is altered in response to exposure to environmental contaminants. Cricket frogs were collected over a three-year period (1993 - 1995) in various locations throughout the state of Illinois (Reeder *et al.*, 1998). Of the five sites where intersex organisms were found, four had detectable atrazine. According to the authors, the relationship between the detection of atrazine and prevalence of intersex approached a significant relation. While a wide range of chemical residue analyses were conducted, only atrazine data were reported. The authors suggested that there may be a trend between atrazine concentrations and the proportion of animals exhibiting intersex.

In Thailand, atrazine has also been detected in aquatic environments but no data regarding the effects of atrazine on amphibians available. Thus, this study was to determine the effects of atrazine on gonadal development by using *H. rugulosus* as a model. The nominal concentrations were 0.001 to 1,000 ppb, which is the range of concentration of atrazine residues reported by the U.S. EPA (Dorsey *et al.* 2003).

Materials and methods

Mature males and females *H. rugulosus* were maintained separately in 500 liter plastic tanks containing de-chlorinated tap water and fed once a day with frog chow, Higrad^{TM}. Breeding were induced by injection of human chorionic gonadotropin analogue (Hauy Hong Krai Royal Development Study Centre, 2002). After eggs were laid, the females and the males were removed.

Fertilized eggs were reared in 20 liters glass tanks, incubated at 25 ± 2 °C with 12-hours light/ 12-hours dark cycle. Animal were reared with 4 liter Holtfreter's solutions. On day 3 after fertilization, larvae were fed with frog chow, Higrade™, *ad libitum*. The normal table of 66 stages of *Xenopus laevis* (Nieuwkoop and Faber, 1994) was used as references to determine stages. Embryos were exposed to atrazine with nominal concentrations of 0, 0.001, 0.1, 1, 10, 25, 100, and 100 ppb atrazine respectively (98% pure; Chemservice, Chester, PA). Atrazine was pre-dissolved in ethanol, and all treatments contained 0.025% ethanol. Each treatment was replicated three times (30 larvae/replicate). Static renewal experiment was chosen, so the solutions were renewed every 3 days. All treatments were randomly relocated every 3 days to ensure that no treatments experienced positional effects. Animals were exposed throughout the larval period from fertilization until complete metamorphosis.

At the end of metamorphosis, frogs were anesthetized by 5% MS-222 (3-aminobenzoic acid ethyl ester) then weighed, measured for snout-vent length. The gross gonadal abnormalities were investigated under the dissecting microscope and photographed. The abnormalities were defined as follows; hermaproditism (mix sex), discontinuous of testis, disappearance of gonad (Hayes *et al.* 2002a). The observed gonads were fixed in Bouin's solution for 24 hour then preserved in 70% alcohol.

Histological analysis was performed by sectioning and staining with Haematoxylin and Eosin following method described by Bancroft and Gamble, 2002.

Sex ratio, weight, snout-vent length among controls and treatments were test for statistical analysis. All data were test for normal distribution and homogeneity of variance. For the sex ratio, the Chi-square was applied for the differences between control and treatment. Student t- test was used for the difference between control and treatment for weight, snout-vent length. Parameters that failed the normality test were compared by the Kruskal-Wallis One-way Analysis of Variance test. A significant difference is reported at $p<0.05$. Statistical analysis was carried out using SigmaStat 3.5® for Windows.

Results

The temperature and pH in all condition range from 24.5-26 ºC and 6.0-7.5, respectively. The animal reached the end of metamorphosis within 40-45 days. The percent mortality, snout-vent length, weight, sex ratio, and gonad abnormality were reported in Table 5-1.

The mortality of both solution and ethanol controls and all treatments ranged from 38.88 to 47.77%. The highest mortality rate was in the metamorphic climax or the tail resorption stage which accounted for 80% of the death observed (data not shown) and the rest of death caused by the cannibalism. The statistical analysis revealed that there were no statistical difference among the controls and treatments (Kruskal-Wallis One-way Analysis of Variance, $p<0.05$)

Snout-vent lengths of froglets from all groups ranged from 20.87 to 23.65 mm. There were also no statistical difference among the controls and treatments (ANOVA, $p<0.05$). The weight of froglets ranged from 1.416 to 1.588 g. Statistical analysis of the weights also revealed no statistical difference among the controls and treatments (ANOVA, $p<0.05$)

Sex - ratio of the froglets were studied from morphology of the gonads after dissection. There were more female froglets than the males in all groups, and the sex-ratio between male and female ranged from 1:2.60 to 1:4.00. Chi-square test to compare sex - ratio between the controls and treatments showed no statistical differences of among all groups.

Moreover, the gonads morphology of both male and female froglets observed did not show any morphological abnormalities (*i.e.*, hermaproditism, discontinuous

gonad, disappearance of gonad). The testes were defined by rod shape without lobule (Figure 5-6) and ovaries were defined by lobule shape (Figure 5-7). Histological studies to determine any abnormalities at tissues levels were also carried out. However, no testicular oocyte which is a sign of male gonad tissue abnormality was found in this study.

Table 5-1: The mortality, snout-vent length, weigh, and sex ratio in each group at the end of metamorphosis of *H. rugulosus* .

Concentrations (ppb)	Mortality (percent±SEM)	Snout-vent length (mm±SEM)	Weight (g±SEM)	Sex ratio (male : female)
Control	41.11 ± 2.22	21.96 ± 0.87	1.55 ± 0.08	1 : 3.25
Ethanol control	43.33 ± 3.33	21.95 ± 0.92	1.57 ± 0.06	1 : 2.80
0.001	42.22 ± 7.29	22.79 ± 1.46	1.59 ± 0.03	1 : 3.00
0.01	46.66 ± 1.93	22.02 ± 1.14	1.59 ± 0.02	1 : 3.25
0.1	42.22 ± 1.11	22.17 ± 0.55	1.45 ± 0.08	1 : 2.60
1	43.33 ± 3.33	23.65 ± 1.25	1.42 ± 0.15	1 : 3.00
10	41.11 ± 1.11	23.14 ± 0.52	1.51 ± 0.05	1 : 3.25
25	42.22 ± 4.01	22.59 ± 0.84	1.52 ± 0.05	1 : 4.00
100	38.88 ± 1.11	22.52 ± 0.92	1.54 ± 0.08	1 : 2.60
1,000	47.77 ± 4.84	20.87 ± 0.31	1.43 ± 0.18	1 : 3.33

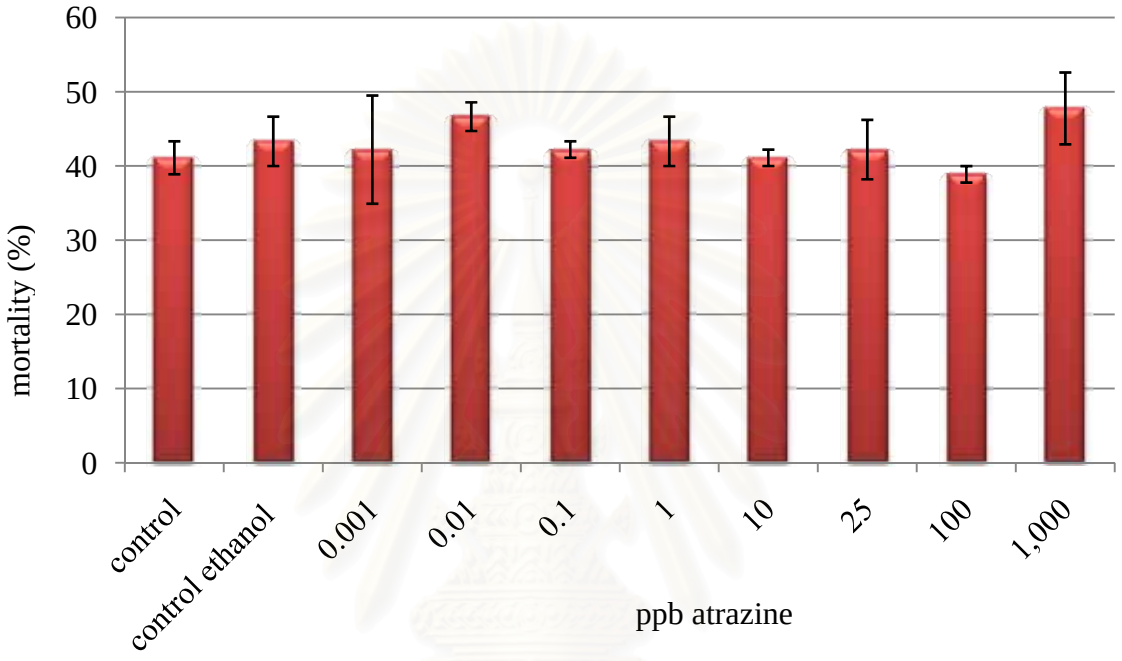

Figure 5-1: Accumulated mortality (%) of *H. rugulosus* in each group at end metamorphosis.

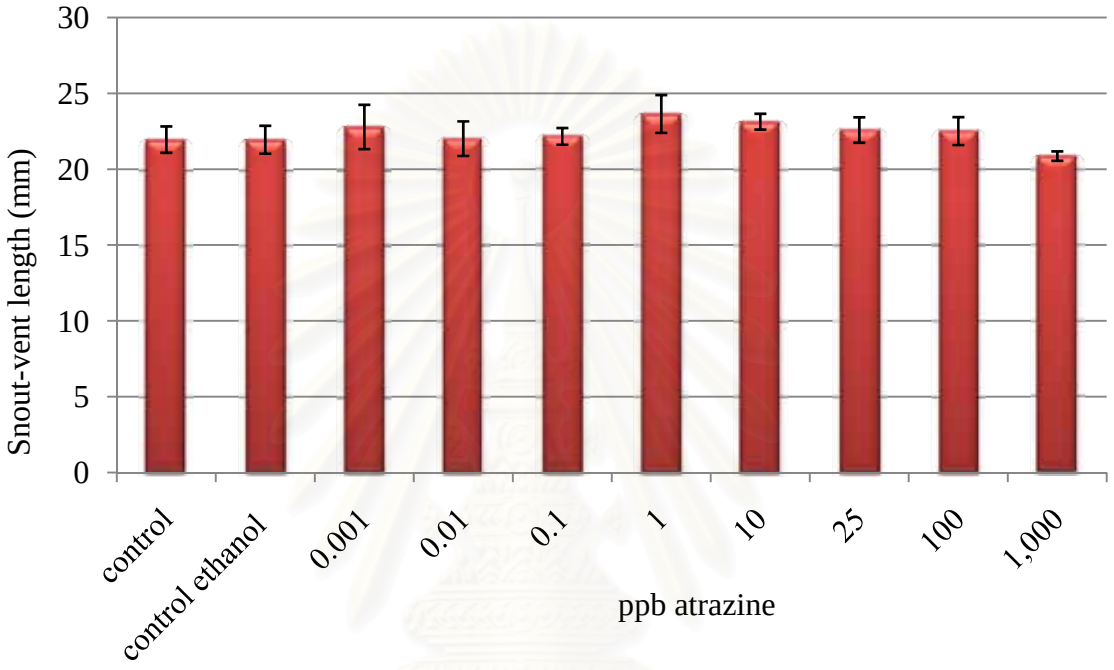

Figure 5-2: Snout-vent length (mm) of *H. rugulosus* in each group at end of metamorphosis.

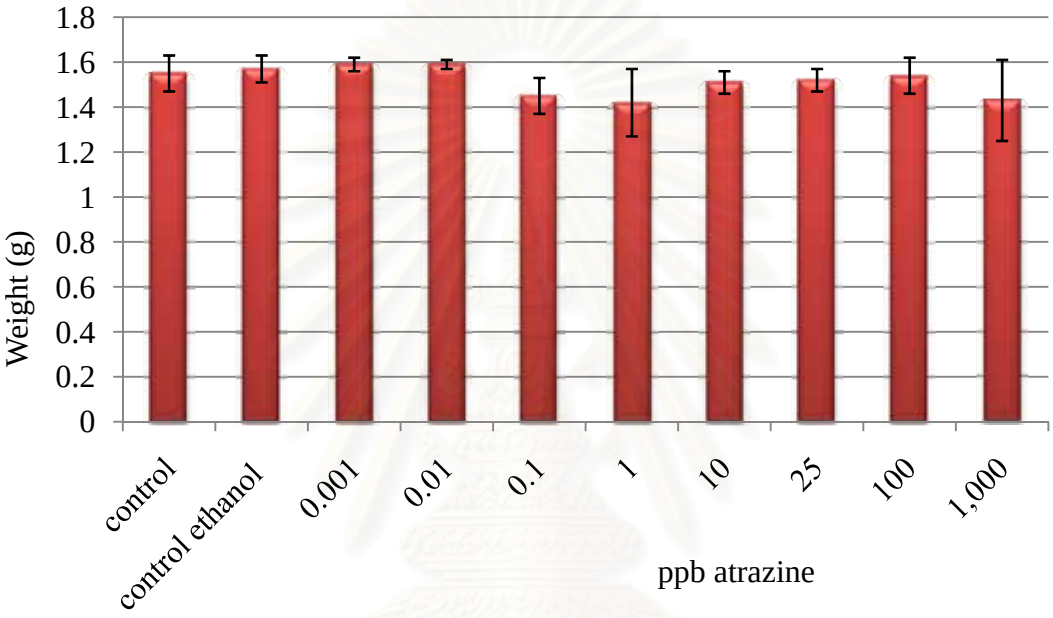

Figure 5-3: Weight (g) of *H. rugulosus* in each group at end of metamorphosis.

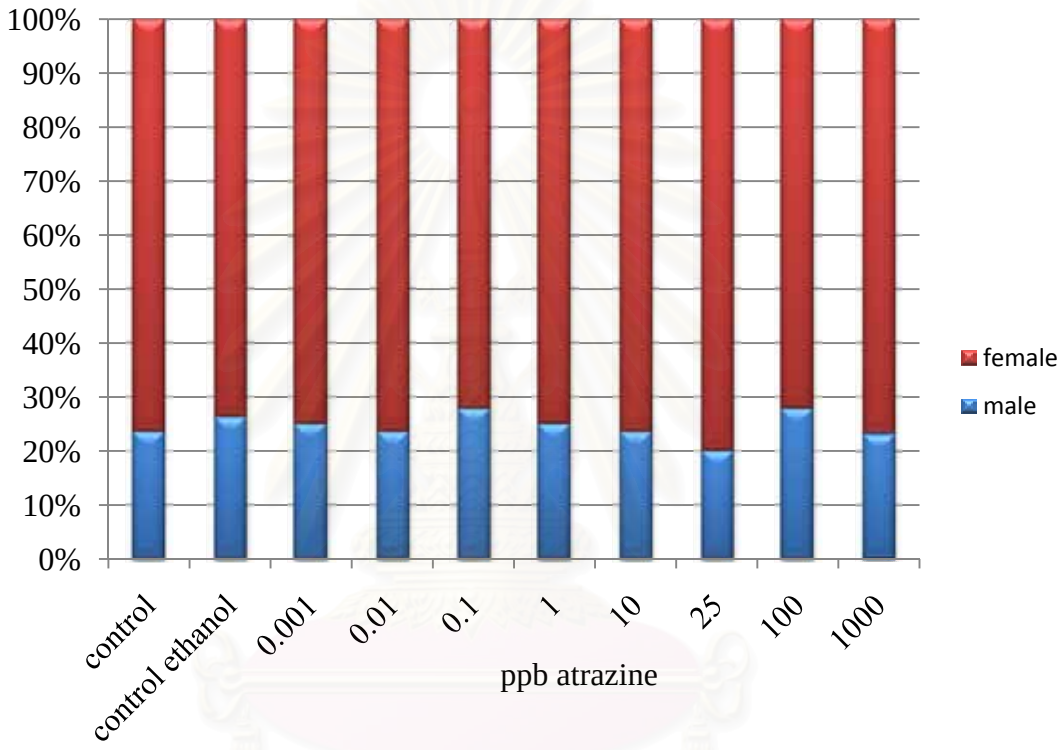

Figure 5-4: Percentage of each sex of *H. rugulosus* in each group at end of metamorphosis.

Figure 5-5: *H. rugulosus* at the end of metamorphosis,

Nieuwkoop and Faber stage 66. (bar = 10 mm)

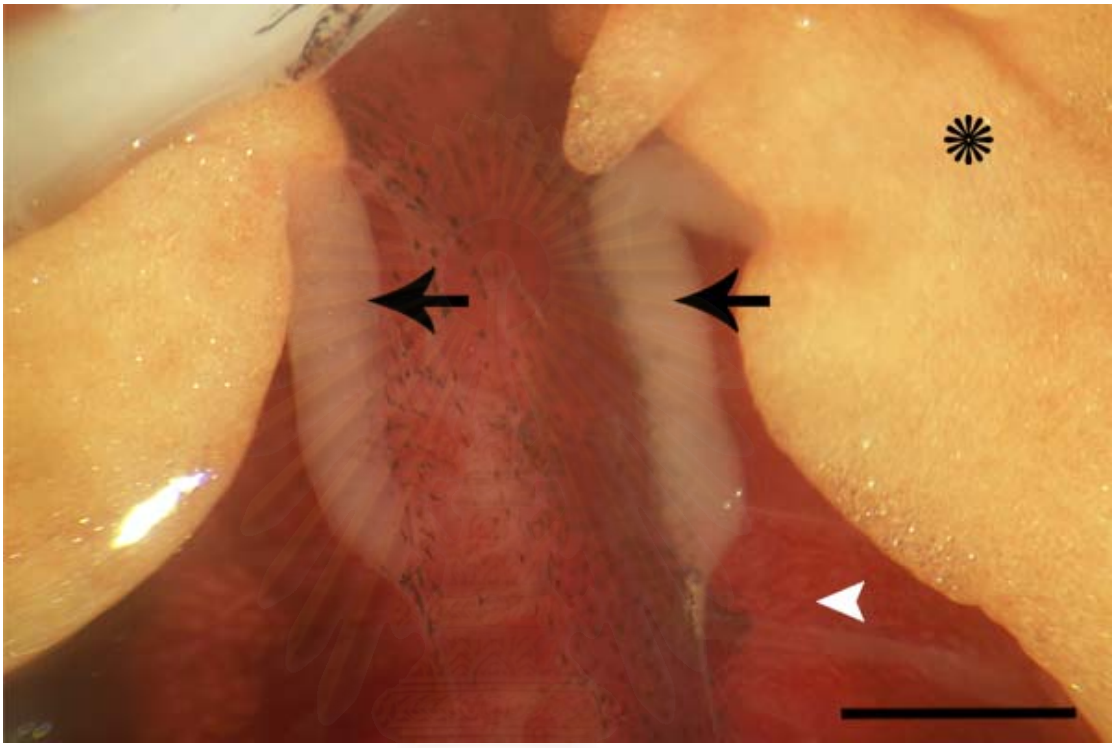

Figure 5-6: Male gonad morphology of *H. rugulosus* at the end of metamorphosis, (arrow = testis, arrow head = kidney, asterisk = fat body, bar = 1 mm).

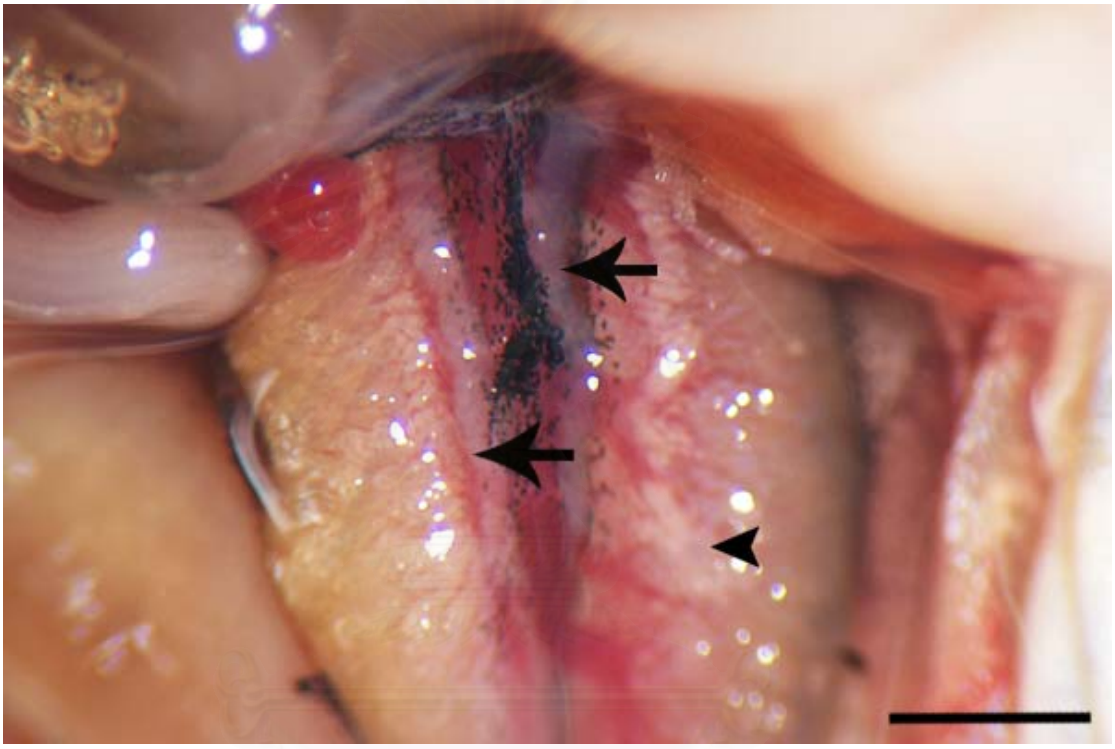

Figure 5-7: Female gonad morphology of *H. rugulosus* at the end of metamorphosis, (arrow = ovary, arrow head = kidney, bar = 1 mm).

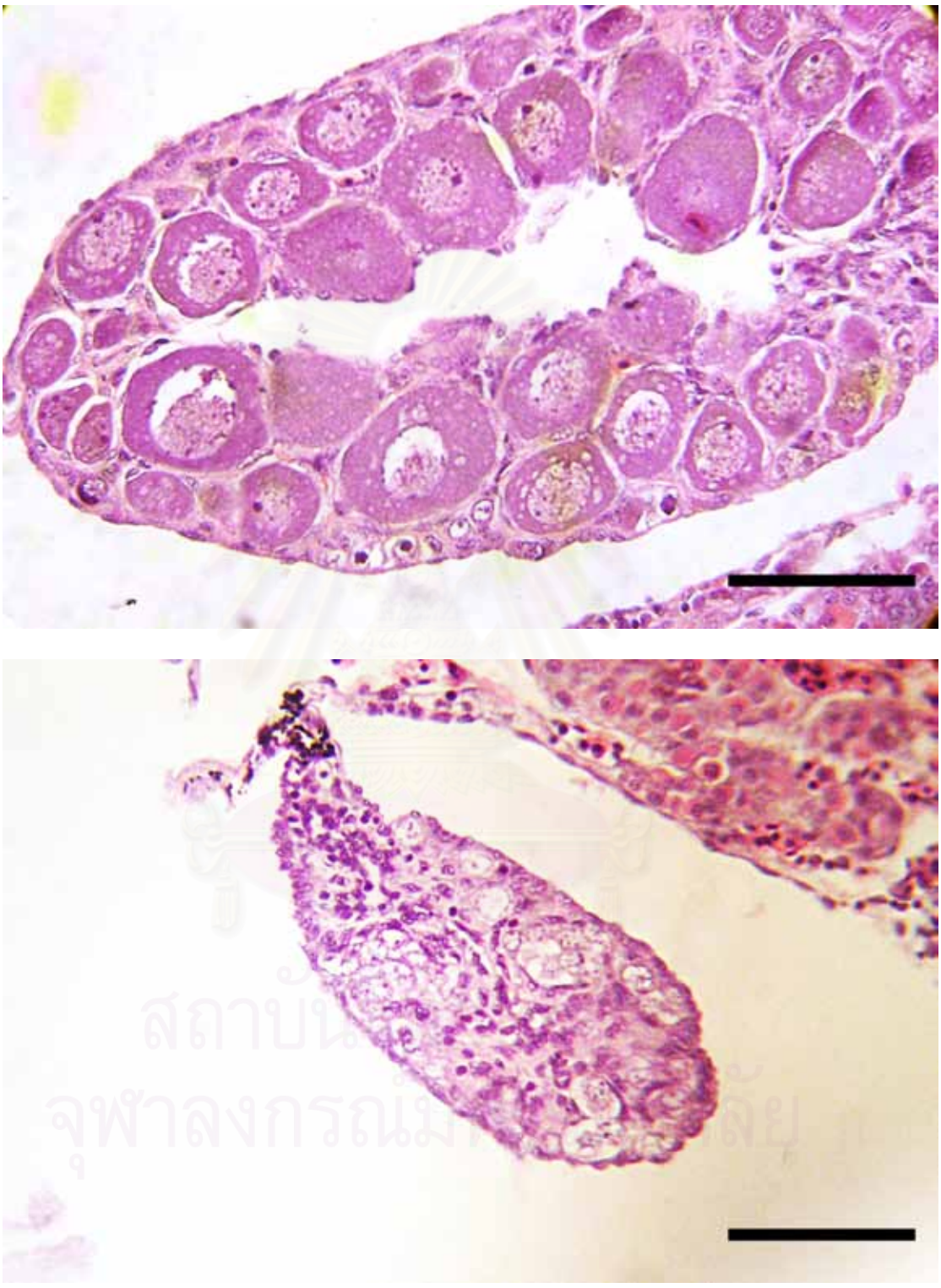

Figure 5-8: Female (a) and male (b) gonad histology of *H. rugulosus* at the end of metamorphosis, (bar = 50 µm).

Discussion

Regarding the fact that there were no significant differences found in mortality, snout-vent length and weight of *H. rugulosus,* it could be concluded that the nominal concentration of atrazine (0.001-1,000 ppb) could not cause the acute death response nor developmental abnormalities in *H. rugulosus*. These concentrations were lower than the acute concentrations reported by previous studies.

Previous studies suggested that atrazine is an endocrine disruptor for amphibians. Hayes *et al*. 2002a first reported the effect of atrazine altering gonad development in *X. laevis* in all the concentrations tested *i.e.* multiple gonads and hermaphroditism. Moreover, the report also showed that male frogs exposed to atrazine higher than 1 ppb had reduced laryngeal dilator muscle size (demasculinization). Additional studies published by Hayes *et al.* (2002b, 2003) reported that similar effects on gonadal development were achieved using the non-native *X. laevis* and the native leopard frog (*Rana pipiens*). Hayes *et al*. (2006) proposed mechanisms of atrazine action inducing aromatase activity resulting in a decrease in androgens (aromatase substrate) and a subsequent increase in estrogens (aromatase product) thus atrazine could demasculinizes and feminizes amphibians.

In contrast, the U.S. EPA (2003) reaches the conclusion that the results of atrazine regarding the hermaphrodite found were uncertain, not reproducible and show no dose-response relationship. The U.S. EPA reviewed the studies from Hayes *et al*. 2002a, Goleman and Carr 2003, Hayes *et al*. 2002c and Du Preez *et al*. 2003 and suggest that the intersex may have occurred due to the unfavorable solution or the inappropriate conditions (Steger and Tietge 2003). Moreover, the field study also found the evidences of hermaproditism, however, there were no significant data to

confirmed that the hermaphroditism found correlated with the used of atrazine (Du Preez *et al*. 2003, Smith *et al*. 2003). Thus, from these conclusions, the U.S. EPA was still allowed to use the atrazine in the U.S.

Oka *et al*. (2008) conducted the experiment using two different groups of *X. laevis* wild-type with mixed in sex and tadpoles and a group of all-male tadpoles. The tadpoles were exposed to concentrations of atrazine that ranged from 0.1 to 100 ppb. They also found no hermaphrodite frogs nor the increase in aromatase as measured by aromatase mRNA induction and also no increase in vitellogenin, another marker of feminization.

Du Preez *et al*. 2008 conducted the experiment to determine whether atrazine (0, 1, 10, 25 ppb) could effects the long term (2 years post-metamorphosis) exposed frogs *X. laevis* by measuring clutch size and survival of offspring both F_1 and F_2. The results show that there were no effects of any of the studied concentrations of atrazine on clutch size of F_1 frogs. There were also no effects on hatching success or time to metamorphosis. Sex ratios did not differ between F_2 offspring among treatments. There was no evidence to suggest a trans-generational effect of atrazine on spawning success or reproductive development of *X. laevis*. Goleman *et al*. (2003) also observed the hermaphroditism in *X. lavae* exposed to estradiol or 25 ppm atrazine. Thus, Renner (2008) suggests that those *X. laevis* gonad abnormalities cause by atrazine experiment ‘aren’t reproducible’.

This study found no evidence of gonad abnormalities. The possible explanations were that atrazine might not effect to this frog species, *H. rugulosus* or the concentrations in this study were not high enough for inducing significant effects. However, to evaluate the effects of atrazine in detail, the aromatase activities and

estrogen level should be observed to confirm that atrazine has no effect on *H. rugulosus*.

In conclusion, the present study found that the nominal concentration of atrazine used did not have any effect on the gonad development of *H. rugulosus*. Therefore, it is suggested that pure atrazine at concentration used in this study could not cause gonad abnormalities in *H. rugulosus*.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

This study used *H. rugulosus* as a experimental animal. Compared with *X. laevis* which is an animal model in the U.S. and Europe, our study suggests that this frog is a potentially alternative experimental model for the following reasons: the adult can be easily reared and reproduced and the embryos also developed satisfactorily in the laboratory. Therefore, *H. rugulosus* could be considered as a suitable representative model for anurans in South East Asia where they are widely distributed. However, due to the lack of data available for this species especially the detailed development of organs and also their molecular developmental aspects, more in depth studies are needed.

This experiment has successfully produced a normal table for embryonic and larval development of *H. rugulosus* which could be used as a reference in further experiments.

The experiments on the effects of atrazine on the embryonic and larval development of *H. rugulosus* have been investigated using the FETAX protocol. The results showed no significant effects on the mortality among the groups. The results are in accordance with (U.S. EPA, 2006) where both acute and chronic treatments of atrazine confirmed that exposure to the nominal concentrations of atrazine used in this study does not directly cause mortality for aquatic organisms.

Even though the results found no statistical differences of the nominal concentrations of atrazine on the embryonic and larval development of *H. rugurosus*, there were certain morphological abnormalities which were found only in the treated groups but not in the control groups. Therefore, these effects of atrazine could not be

neglected. Other reports found similar abnormalities in *X. laevis,* treated with atrazine at concentrations higher than 1.1 ppm (Morgan *et al.* 1996), higher than 5 ppm (Fort *et al.* 2004). Lenkovski *et al*. (2008) found abnormalities in larvae stage 46-49 treated at 25 -50 ppm. However, all these concentrations are evidently higher than what we used in this experiment. The more in depth study concerning the cellular and molecular mechanisms of atrazine on embryonic and larval development might somehow able to help in explaining the observed results.

At present, the explanation for the abnormalities found in the present experiment might be addressed by the genetic make-up of the animals which could have caused them to become more susceptible to 1,000 times lower atrazine concentration than the above mentioned reports.

Atrazine is considered as an endocrine disruptor which alters hormone production not only in experimental animals such as mice and rats but also in the field (U.S. EPA, 2006). Although Hayes *et al*. (2002a, 2006) had observed endocrine disruptor effects of atrazine on anurans, the mechanism for these results have not yet been elucidated, but he proposed that atrazine might interfere with aromatase activities. The induction of aromatase resulted in a decrease in androgens, which are aromatase substrates and an increase in estrogens, the aromatase products. The increase in estrogens in genetic males would induce the emergence of gonads with female characteristics such as the presence of oocytes in the testis, intersex gonads or hermaphrodite conditions. Present study followed the procedure used by Hayes *et al.* (2002), except *X. laevis* and *R. pipiens* were substituted by *H. rugulosus*. However, neither gonad abnormalities nor hermaproditism were observed in this experiment. It could be possible that there is species different in response to atrazine.

The effect of atrazine at ecologically relevant levels on gonads is an on-going controversy. The U.S. EPA has not accepted the lower concentration effects with one major reason being that the tests are not reproducible and 'currently insufficient data' are available. Furthermore, regarding experiments in the laboratory, the U.S. EPA concluded that the effects observed did not occur in dose response patterns. In field studies which found gonad abnormalities in frogs caught in the wild, the U.S. EPA also suggested that these abnormalities had no correlation with conventional dose response fashion.

The U.S. EPA summarized that, "while the current research does not support a definitive conclusion regarding a quantitative dose-response relationship between atrazine exposure and effects on gonad development, it provides sufficient information to formulate a hypothesis that atrazine exposure may affect gonad development."

However, the European Union (EU - EPHA, 2008) has withdrawn its approval to atrazine and it is banned in many European countries as many amphibian abnormalities were found in natural environments where atrazine was detected.

Nevertheless, even if this study concludes that nominal concentrations of atrazine do not affect the gonad development of *H. rugulosus*, the abnormalities observed in this study and previous must not be neglected. This study would suggest that it is only pure atrazine that does not produce gonad abnormalities.

In agriculture many chemicals are used synergistically in farmland including fertilizers, pesticides and other chemicals which can cause contamination on aquatic environments. Mixed contaminations are commonly observed and aquatic organisms could be suffering from these combined effects. Furthermore, the mixture of these chemicals could interfere with other hormone systems apart from the reproductive

hormones. Thus not only the reproductive system but also other systems which are controlled by their respective hormones could also suffer these changes. Consequently many hormones affected by these endocrine disruptors could affect the development of aquatic animals including amphibian larvae. At present even if atrazine is still permitted in the U.S., Thailand and many other countries, researchers have to carefully reconsider the combined effects of atrazine and other chemicals on aquatic organisms.

In conclusion, atrazine is still permitted in many countries with previous studies showing no concrete evidence of its effects on amphibians and others species. Although these manifestations have not been accepted as a ‘conventional’ toxicity response or dose response, it cannot conclude that no effects of atrazine were observed, even if it is less statistically acceptable. In real agricultural practice atrazine and other chemicals are applied in frequent and high concentrations which could affect many aquatic organisms. These dosages are certainly higher, (even if they are not scientifically measured), than the ‘ecologically relevant doses’ measured after run-off. Therefore, the aquatic organisms in the field including amphibians could suffer directly from high concentrations of atrazine and also the combined effects with other agro-chemicals which may partly explain the amphibian decline.

Finally, this study would suggest that experiments which closely imitate the practiced agricultural conditions should be performed to better elucidate the effects of agrochemicals on non-target organisms including amphibians.

References

Alazemi, B. M., Lewis, J. W. and Andrews, E. B. 1996. Gill damage in the freshwater fish *Gnathonemus petersii* (family: Mormyridae) exposed to selected pollutants: An ultrastructural study. Environmental Technology 17(3): 225-238.

Albanis, T. A., Hela, D. G., Sakellarides, T. M. and Konstantinou, I. K. 1998. Monitoring of pesticide residues and their metabolites in surface and underground waters of Imathia (N. Greece) by means of solid-phase extraction disks and gas chromatography. Journal of Chromatography-part A 823(1-2): 59-71.

Allran, J. W. and Karasov, W. H. 2001. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environmental Toxicology *and* Chemistry 20(4): 769-75.

Aso, S., Anai, M., Noda, S., Imatanaka, N., Yamasaki, K. and Maekawa, A. 2000. Twenty-Eight-Day Repeated-Dose Toxicity Studies for Detection of Weak Endocrine Disrupting Effects of Nonylphenol and Atrazine in Female Rats. Journal of Toxicological Pathology 13(1): 13.

ASTM Standard E1439-98 (2004). Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). ASTM International, West Conshohocken, PA.

Baillie, J. E. M., Bennun, L. A., Brooks, T. M., Butchart, S. H. M., Chanson, J. S., Cokeliss, Z., Hilton-Taylor, C., Hoffmann, M., Mace, G. M., Mainka, S. A., Pollock, C. M., Rodrigues, A. S. L., Stattersfield, A. J. and Stuart, S. N. 2004. 2004 IUCN Red List of Threatened Species, A Global Species Assessment. IUCN, Gland, Switzerland and Cambridge, UK.

Battaglin, W. A. and Goolsby, D. A. 1999. Are shifts in herbicide use reflected in concentration changes in midwestern Rivers?. Environmental Science & Technology 33(17): 2917-2925.

Beebee, T. J. C. and Griffiths, R. A. 2005. The amphibian decline crisis: A watershed for conservation biology?. Biological Conservation 125(3): 271-285.

Bester, K. and Huhnerfuss, H. 1993. Triazines in the Baltic and North Sea. Marine Pollution Bulletin 26(8): 423-427.

Birge, W. J., Black, J. A., Westerman, A. G. and Ramey, B. 1983. Fish and amphibian embryos: A model system for evaluating teratogenicity. Fundamental and Applied Toxicology 3(4): 237-242.

Boone, M. D. and James, S. M. 2003. Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. Ecological Applications 13(3): 829-841.

Brooke, L. 1990. University of Wisconsin-Superior, Superior, WI. (Memorandum to R. L. Spehar, US. EPA, Duluth, MN. January 30). .cited in Dorsey, A., Little, S. S., Knaebel, D. B., Plewak, D., Printup, H. and Rhoades, J. (2003). Toxicological Profile for Atrazine. U.S. Department of Health and Human Survice, Public Health Service Agency for Toxic Substances and Disease Registry. Available from: www.epa.gov

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., Solomon, K. R. and Van Der Kraak, G. 2003. Response of larval *Xenopus laevis* to atrazine: Assessment of growth, metamorphosis, and gonadal and laryngeal morphology. Environmental Toxicology and Chemistry 22(2): 396-405.

Coady, K., Murphy, M., Villeneuve, D., Hecker, M., Jones, P., Carr, J., Solomon, K., Smith, E., Van Der Kraak, G., Kendall, R. and Giesy, J. 2004. Effects of atrazine on metamorphosis, growth, and gonadal development in the green frog (*Rana clamitans*). Journal of Toxicology and Environmental Health part A 67(12): 941-57.

Cooper, R. L., Stoker, T. E., Goldman, J. M., Parrish, M. B. and Tyrey, L. 1996. Effect of atrazine on ovarian function in the rat. Reproductive Toxicology 10(4): 257-264.

Cooper, R. L., Stoker, T. E., Tyrey, L., Goldman, J. M. and McElroy, W. K. 2000. Atrazine Disrupts the Hypothalamic Control of Pituitary-Ovarian Function. Toxicological Sciences 53(2): 297-307.

Crain, D. A., Guillette Jr, L. J., Rooney, A. A. and Pickford, D. B. 1997. Alterations in steroidogenesis in alligators (*Alligator mississippiensis*) exposed naturally and experimentally to environmental contaminants. Environmental Health Perspectives 105(5): 528-533.

Davies, P. E., Cook, L. S. J. and Barton, J. L. 1994. Triazine herbicide contamination of Tasmanian streams: sources, concentrations and effects on biota. Australian Journal of Marine & Freshwater Research 45(2): 209-226.

De Solla, S. R., Martin, P. A., Fernie, K. J., Park, B. J. and Mayne, G. 2006. Effects of environmentally relevant concentrations of atrazine on gonadal development of snapping turtles (*Chelydra serpentina*). Environmental Toxicology and Chemistry 25(2): 520-526.

Del Conte, E. and Sirlin, J. L. 1952. Pattern series of the first embryonary stages in *Bufo arenarum.* The Anatomical Record 112(1): 125-135.

Department of Agriculture Thailand. 1998. Import data of agricultural goods in Thailand,1998. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Department of Agriculture Thailand. 1999. Import data of agricultural goods in Thailand,1999. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Department of Agriculture Thailand. 2000. Import data of agricultural goods in Thailand, 2000. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Department of Agriculture Thailand. 2001. Import data of agricultural goods in Thailand, 2001. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Department of Agriculture Thailand. 2002. Import data of agricultural goods in Thailand, 2002. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Department of Agriculture Thailand. 2003. Import data of agricultural goods in Thailand, 2003. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Department of Agriculture Thailand. 2004. Import data of agricultural goods in Thailand, 2004. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Department of Agriculture Thailand. 2005. Import data of agricultural goods in Thailand, 2005. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Department of Agriculture Thailand. 2006. Import data of agricultural goods in Thailand, 2006. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Department of Agriculture Thailand. 2007. Import data of agricultural goods in Thailand, 2007. Department of Agriculture, Ministry of Agricultures and Cooperatives, Thailand. Available form: www.doa.go.th

Diana, S. G., Resetarits Jr, W. J., Schaeffer, D. J., Beckmen, K. B. and Beasley, V. R. 2000. Effects of atrazine on amphibian growth and survival in artificial aquatic communities. Environmental Toxicology and Chemistry 19(12): 2961-2967.

Diesmos, A., Dijk, P. P. v., Inger, R., Iskandar, D., Lau, M. W. N., Ermi, Z., Shunqing, L., Baorong, G., Kuangyang, L., Zhigang, Y., Huiqing, G., Haitao, S. and Wenhao, C. (2004). *Hoplobatrachus rugulosus*. In: IUCN 2008. , 2008 IUCN Red List of Threatened Species. Downloaded on 30 March 2009

Dorsey, A., Little, S. S., Knaebel, D. B., Plewak, D., Printup, H. and Rhoades, J. (2003). Toxicological Profile for Atrazine. U.S. Department of Health and Human Survice, Public Health Service Agency for Toxic Substances and Disease Registry. Available from: www.epa.gov

Du Preez, L. H., Kunene, N., Everson, G. J., Carr, J. A., Giesy, J. P., Gross, T. S., Hosmer, A. J., Kendall, R. J., Smith, E. E., Solomon, K. R. and Van Der Kraak, G. J. 2008. Reproduction, larval growth, and reproductive development in African clawed frogs (*Xenopus laevis*) exposed to atrazine. Chemosphere 71(3): 546-552.

Eldridgea, J. C., Wetzel, L. T. and Tyrey, L. 1999. Estrous cycle patterns of Sprague-Dawley rats during acute and chronic atrazine administration. Reproductive Toxicology 13(6): 491-499.

U.S. Environmental Protection Agency. (2005). Atrazine Chemical Summary. United States Environmental Protection Agency. Available form: www.epa.gov

U.S. Environmental Protection Agency. (2006). Effect Determination for Atrazine. U.S. Environmental Protection Agency. Available form: www.epa.gov

Fischer-Scherl, T., Veeser, A., Hoffmann, R. W., Kuhnhauser, C., Negele, R. D. and Ewringmann, T. 1991. Morphological effects of acute and chronic atrazine exposure in rainbow trout (*Oncorhynchus mykiss*). Archives of Environmental Contamination and Toxicology 20(4): 454-461.

Fort, D. J., Rogers, R. L., Thomas, J. H., Buzzard, B. O., Noll, A. M. and Spaulding, C. D. 2004. Comparative sensitivity of *Xenopus tropicalis* and *Xenopus laevis* as test species for the FETAX model. Journal of Applied Toxicology 24(6): 443-457.

Frank, R. and Sirons, G. J. 1985. Dissipation of atrazine residues from soils. Bulletin of Environmental Contamination and Toxicology 34(1): 541-548.

Gaynor, J. D., Tan, C. S., Drury, C. F., van Wesenbeeck, I. J. and Welacky, T. W. 1995. Atrazine in surface and subsurface runoff as affected by cultural practices. Water Quality Research Journal of Canada 30(3).

Glotfelty, D. L., Leech, M. M., Jersey, J. and Taylor, A. W. 1989. Volatilization and wind erosion of soil surface applied atrazine, simazine, alachor, and toxaphene. Journal of Agricultural and Food Chemistry 37.

Gorge, G. and Nagel, R. 1990. Toxicity of lindane, atrazine, and deltamethrin to early life stages of zebrafish (*Brachydanio rerio*). Ecotoxicology and Environmental Safety 20(3): 246-255.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16(3): 183-190.

Hauy Hong Krai Royal Development Study Centre. 2002. Frog farming. Hauy Hong Krai Royal Development Study Centre, Doi Saked district, Chainmai, Thailand. (in Thai)

Hayes, T. B. 1998. Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. Journal of Experimental Zoology 281(5): 373-99.

Hayes, T. B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V. P., Marjuoa, Y., Parker, J. and Tsui, M. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact?. Environmental Health Perspectives 114 Suppl 1: 40-50.

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A. and Vonk, A. 2002a. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the

National Academy of Sciences 99(8): 5476-80.

Hayes, T. B., Haston, K., Tsui, M., Hoang, A., Haeffele, C. and Vonk, A. 2002b. Herbicides: feminization of male frogs in the wild. Nature 419(6910): 895-6.

Hayes, T. B., Haston, K., Tsui, M., Hoang, A., Haeffele, C. and Vonk, A. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environmental Health Perspectives 111(4): 568-75.

Hayes, T. B. and Menendez, K. P. 1999. The effect of sex steroids on primary and secondary sex differentiation in the sexually dichromatic reedfrog (*Hyperolius argus*: Hyperolidae) from the Arabuko Sokoke Forest of Kenya. General and Comparative Endocrinology 115(2): 188-99.

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 (17beta-estradiol): Support for the demasculinization/feminization hypothesis. Environmental Health Perspectives 114 Suppl 1: 134-41.

Hussein, S. Y., El-Nasser, M. A. and Ahmed, S. M. 1996. Comparative studies of the effects of herbicide atrazine on freshwater fish *Oreochromis niloticus* and *Chrysichthyes auratus* at Assiut, Egypt. Bulletin of Environmental Contamination and Toxicology 57(3): 503-510.

IUCN (2004). Global Amphibian Assessment. Available from: www.iucn.org

Johnson, P. T. J., Chase, J. M., Dosch, K. L., Hartson, R. B., Gross, J. A., Larson, D. J., Sutherland, D. R. and Carpenter, S. R. 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proceedings of the National Academy of Sciences of the United States of America 104(40): 15781-15786.

Kettle, W. D., DeNoyelles Jr, F., Heacock, B. D. and Kadoum, A. M. 1987. Diet and reproductive success of bluegill recovered from experimental ponds treated with atrazine. Bulletin of Environmental Contamination and Toxicology 38(1): 47-52.

Lenkowski, J. R., Reed, J. M., Deininger, L. and McLaughlin, K. A. 2008. Perturbation of organogenesis by the herbicide atrazine in the amphibian *Xenopus laevis*. Environmental Health Perspectives 116(2): 223-30.

McDiarmid, R. W. and Altig, R., Ed. 1999. Tadpoles: the Biology of Anuran Larvae. University of Chicago Press. Illinois, USA

Ministry of Commerce Thailand. 2008. The potentially agricutural export goods 2007-2008. Ministry of Commerce Thailand. Available from: www.moc.go.th

Moore, A. and Waring, C. P. 1998. Mechanistic effects of a triazine pesticide on reproductive endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr. Pesticide Biochemistry and Physiology 62(1): 41-50.

Morgan, M. K., Scheuerman, P. R., Bishop, C. S. and Pyles, R. A. 1996. Teratogenic potential of atrazine and 2,4-D using FETAX. Journal of Toxicology and Environmental Health - Part A 48(2): 151-168.

Neskovic, N. K., Elezovic, I., Karan, V., Poleksic, V. and Budimir, M. 1993. Acute and subacute toxicity of atrazine to carp (*Cyprinus carpio* L.). Ecotoxicology and Environmental Safety 25(2): 173-182.

Nieuwkoop, P. D. and Faber, J. 1994. Normal Table of *Xenopus Laevis* (Daudin): A Systematical & Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis. 1st ed. Garland Science

Oka, T., Tooi, O., Mitsui, N., Miyahara, M., Ohnishi, Y., Takase, M., Kashiwagi, A., Shinkai, T., Santo, N. and Iguchi, T. 2008. Effect of atrazine on metamorphosis and sexual differentiation in *Xenopus laevis*. Aquatic Toxicololgy 87(4): 215-26.

Pollister, A. W. and Moore, J. A. 1937. Tables for the normal development of *Rana sylvatica*. The Anatomical Record 68(4): 489-496.

Pollution Control Department Thailand. 2000. Report of the laws of pollution control in Thailand. Ministry of Natural Resources and Environment, Pollution Control Department. Available from: www.pcd.go.th

Poonsuk Haruetaithanasant (2005). Report of the researches of Poonsuk Haruetaithanasant. Department of Agriculture, Ministry of Agriculture and Cooperatives. (in Thai)

Reeder, A. L., Foley, G. L., Nichols, D. K., Hansen, L. G., Wikoff, B., Faeh, S., Eisold, J., Wheeler, M. B., Warner, R., Murphy, J. E. and Beasley, V. R. 1998. Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). Environmental Health Perspectives 106(5): 261-266.

Renner, R. 2008. Atrazine effects in *Xenopus* aren't reproducible. Environmental Science and Technology 42(10): 3491-3493.

Rohr, J. R., Elskus, A. A., Shepherd, B. S., Crowley, P. H., McCarthy, T. M., Niedzwiecki, J. H., Sager, T., Sih, A. and Palmer, B. D. 2003. Lethal and sublethal effects of atrazine, carbaryl, endosulfan, and octylphenol on the streamside salamander (*Ambystoma barbouri*). Environmental Toxicology and Chemistry 22(10): 2385-2392.

Rohr, J. R., Elskus, A. A., Shepherd, B. S., Crowley, P. H., McCarthy, T. M., Niedzwiecki, J. H., Sager, T., Sih, A. and Palmer, B. D. 2004. Multiple stressors and salamanders: Effects of an herbicide, food limitation, and hydroperiod. Ecological Applications 14(4): 1028-1040.

Roses, N., Poquet, M. and Munoz, I. 1999. Behavioural and histological effects of atrazine on freshwater molluscs (*Physa acuta* Drap. and *Ancylus fluviatilis* Mull. Gastropoda). Journal of Applied Toxicology 19(5): 351-356.

Shumway, W. 1940. Stages in the normal development of *Rana pipiens* I. External form. The Anatomical Record 78(2): 139-147.

Steeger, T. and Tietge, J. 2003. White Paper on Potential Development Effects of Atrazine on Amphibians. Office of Prevention, Pesticides and Toxic Substances Office of Pesticide Programs Environmental Fate and Effects Division Washington, D.C. Available from: www.epa.gov

Steinberg, C. E. W., Lorenz, R. and Spieser, O. H. 1995. Effects of atrazine on swimming behavior of zebrafish, *Brachydanio rerio*. Water Research 29(3): 981-985.

Stevens, J. T. 1999. A risk characterization for atrazine oncogenicity profile. Journal of Toxicology and Environmental Health- Part A 56(2): 69 - 109.

Stevens, J. T., Breckenridge, C. B., Wetzel, L. T., Gillis, J. H., Luempert Iii, L. G. and Eldridge, J. C. 1994. Hypothesis for mammary tumorigenesis in Sprague-Dawley rats exposed to certain triazine herbicides. Journal of Toxicology and Environmental Health 43(2): 139-153.

Storrs, S. I. and Kiesecker, J. M. 2004. Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. Environmental Health Perspectives 112(10): 1054-7.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L. and Waller, R. W. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306(5702): 1783-1786.

Tavera-Mendoza, L., Ruby, S., Brousseau, P., Fournier, M., Cyr, D. and Marcogliese, D. 2002a. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. Environmental Toxicology *and* Chemistry 21(3): 527-531.

Tavera-Mendoza, L., Ruby, S., Brousseau, P., Fournier, M., Cyr, D. and Marcogliese, D. 2002b. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the ovary. Environmental Toxicology *and* Chemistry 21(6): 1264-1267.

Taylor, A. C. and Kollros, J. J. 1946. Stages in the normal development of *Rana pipiens* larvae. The Anatomical Record 94(1): 7-23.

Taylor, E. H. 1962. The amphibian fauna of Thailand. Kansas University Science Bulletin 43(8).

Thurman, E. M. and Cromwell, A. E. 2000. Atmospheric Transport, Deposition, and Fate of Triazine Herbicides and Their Metabolites in Pristine Areas at Isle Royale National Park. Environmental Science & Technology 34(15): 3079-3085.

Thurman, E. M., Goolsby, D. A., Meyer, M. T. and Kolpin, D. W. 1991. Herbicides in surface waters of the midwestern United States: the effect of spring flush. Environmental Science & Technology 25(10): 1794-1796.

Trevisan, M., Montepiani, C., Ragozza, L., Bartoletti, C., Ioannilli, E. and Del Re, A. A. M. 1993. Pesticides in rainfall and air in Italy. Environmental Pollution 80(1): 31-39.

Trochimowicz, H. J., Kennedy, G. L., Krivanek, N. D. and Ide, H. 2001. Alkyl pyridines and miscellaneous organic nitrogen compounds. Patty's Toxicology. C. B. Bingham E, Powell CH, New York: John Wiley and Sons. **4**. Cited in Dorsey, A., Little, S. S., Knaebel, D. B., Plewak, D., Printup, H. and Rhoades, J. (2003). Toxicological Profile for Atrazine. U.S. Department of Health and Human Survice, Public Health Service Agency for Toxic Substances and Disease Registry. Available from: www.epa.gov

Wacksman, M. N., Maul, J. D. and Lydy, M. J. 2006. Impact of atrazine on chlorpyrifos toxicity in four aquatic vertebrates. Archives of Environmental Contamination *and* Toxicology 51(4): 681-9.

Weisz, P. B. 1945. The normal stages in the development of the South African clawed toad, *Xenopus laevis*. The Anatomical Record 93(2): 161-169.

Wetzel, L. T., Luempert, L. G., 3rd, Breckenridge, C. B., Tisdel, M. O., Stevens, J. T., Thakur, A. K., Extrom, P. J. and Eldridge, J. C. 1994. Chronic effects of atrazine on estrus and mammary tumor formation in female Sprague-Dawley and Fischer 344 rats. Journal of Toxicology and Environmental Health 43(2): 169-82.

Wienhold, B. J. and Gish, T. J. 1994. Effect of Formulation and Tillage Practice on Volatilization of Atrazine and Alachlor. Journal of Environmental Quality 23(2): 292-298.

Wilhelms, K. W., Cutler, S. A., Proudman, J. A., Anderson, L. L. and Scanes, C. G. 2005. Atrazine and the hypothalamo-pituitary-gonadal axis in sexually maturing precocial birds: Studies in male Japanese quail. Toxicological Sciences 86(1): 152-160.

Wilhelms, K. W., Cutler, S. A., Proudman, J. A., Anderson, L. L. and Scanes, C. G. 2006. Effects of atrazine on sexual maturation in female Japanese quail induced by photostimulation or exogenous gonadotropin. Environmental Toxicology and Chemistry 25(1): 233-240.

Young, B. E., Stuart, S. N., Chanson, J. S., Cox, N. A. and Boucher, T. M. (2004). Disappearing Jewels: The Status of NewWorld Amphibians., NatureServe, Arlington, Virginia.

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