Tropical Natural History 20(1): 28–42, April 2020
©2020 by Chulalongkorn University

Multiple Tail-like Structure Induced by Nitrogen Fertilisers in *Hoplobatrachus rugulosus* Embryos

WARET TRACHANTONG[1], MONRUEDEE CHAIYAPO[2,3] AND KANOKPORN SAENPHET[2,3*]

[1]PhD Degree Program in Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, THAILAND
[2]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, THAILAND
[3]Center of Excellence in Bioresources for Agriculture, Industry and Medicine, Chiang Mai University, Chiang Mai, 50200, THAILAND
* Corresponding author. Kanokporn Saenphet (stit.lilo123@gmail.com)
Received: 10 June 2019; Accepted: 1 November 2019

ABSTRACT.– Agrochemical contamination is claimed as one of the most important factors in amphibian decline. Although many researchers previously focused on pesticide toxicity, fertiliser toxicity is also a prominent issue due to the massive amounts applied to fields by farmers each year. Therefore, the present study aimed to investigate acute toxicity of nitrogen fertilisers (ammonium sulphate and urea) on mortality and development of gastrula and neurula in the East Asian bull frog (*Hoplobatrachus rugulosus*) using the frog embryo teratogenesis assay. The results revealed lethality, malformation, and negative developmental effects induced by ammonium sulphate and urea fertilisers in *H. rugulosus* gastrulae and neurulae. Ammonium sulphate produced more severe effects on *H. rugulosus* embryos compared to urea for all measures in the same stage of embryos. Gastrulae were more sensitive to the exposure of the two nitrogen fertilisers. Moreover, the present study is the first report of a multiple tail-like structure caused by fertilisers in frog embryos. The two fertilisers also produced oedema and kinking of tail and body in both stages. This study suggests that the abnormality occurred due to interference with cell movements during gastrulation.

KEY WORDS: ammonium sulphate, urea, *Hoplobatrachus rugulosus*, teratogenesis, multiple tail-like structure

INTRODUCTION

Amphibian populations have been gradually declining for several decades, and species extinction is one of the most serious ecological problems (Houlahan et al., 2000). This crisis has myriad contributing factors, for example, habitat destruction, increase in ultraviolet (UV) radiation and, especially, agricultural pollution (Mann et al., 2009). Even though agrochemical contamination is a crucial issue, previous amphibian studies mainly focused on pesticide toxicity. Toxic effects from over 80 types of agrochemicals in various grades and purities were reported in many species of amphibians (Fryday and Thompson, 2012). However, the toxicity of only a few fertilisers have been documented, especially in frog embryos (Fryday and Thompson, 2012). The median lethal concentration (LC_{50}) value of ammonium nitrate was recorded in various species, including the American toad *Bufo americanus* (60-174 ppm), the Northern leopard frog *Rana pipiens* (100 ppm), and the Western chorus frog *Pseudacris triseriata* (75 ppm). Schuytema and Nebeker (1999) reported that the LC_{50} value of ammonium nitrate in the African clawed frog *Xenopus laevis* embryos is 27.5 mg/L. Nonetheless, the toxicity profile of the remaining fertilisers remain insufficiently characterised. Marco et al. (2001) revealed the sensitivity of the Western redback

salamander *Plethodon vehiculum*, the Southern torrent salamander *Rhyacotriton variegatus* and the rough-skinned newt *Taricha granulosa* to urea fertiliser without a particular lethal concentration. Wijer et al. (2003) disclosed that the exposure to 43-128 ppm calcium nitrate has no effect on survivorship of the European common frog *Rana temporaria*. Although the fertiliser toxicity has only been slightly investigated, farmers apply massive amounts of fertilisers. From 2003 to 2010, the global fertiliser application was defined as at least 64-times greater than pesticides each year (FAO, 2016). As a consequence of this massive usage, nitrogen contamination can be easily detected in aquatic environments. It can trigger eutrophication, hypoxia, toxic algal blooms, and loss of biodiversity (Husk et al., 2017) and affect the decline of the amphibian population. Therefore, the present study aimed to investigate the effect of two nitrogen-based fertilisers (ammonium sulphate and urea) on mortality and development of gastrulae and neurulae in *Hoplobatrachus rugulosus*.

MATERIALS AND METHODS

Animals

The East Asian bull frog (*H. rugulosus*) was used for the experiment in this study. Adult frogs were obtained from a frog farm in Chiang Mai province, Thailand. Acclimatisation of the frogs proceeded under laboratory conditions (21 ± 1°C with 12-h light and 12-h dark photoperiod) for a week before the experiment. A pair of adult frogs were kept in plastic tanks with artificial rain overnight to induce mating. The embryos in gastrula (Gosner stage 10) and neurula (Gosner stage 13) stages were collected for the research (Gosner, 1960).

Chemicals

Commercial-grade ammonium sulphate and urea fertilisers were chosen to examine toxicity in the present study. The ammonium sulphate fertiliser contains 21% nitrogen and 24% sulphur (Parich Fertilizer Co., Ltd., Thailand). The urea fertiliser contains 46% nitrogen and less than 1% biuret (Parich Fertilizer Co., Ltd.). Holtfreter's solution (Holtfreter, 1931) was used as a solvent to prepare the solution of the two nitrogenous fertilisers.

Experimental Design

The experiment was divided into four groups as follows: gastrulae treated with ammonium sulphate fertiliser, neurulae treated with ammonium sulphate fertiliser, gastrulae treated with urea fertiliser, and neurulae treated with urea fertiliser. For groups of treated gastrulae, ammonium sulphate concentrations were 0.01, 0.1, 0.25, 0.5, 0.75 or 1.0 g/L and urea concentrations were 0.1, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5 or 15.0 g/L. For neurula treatment, ammonium sulphate concentrations were 0.46, 0.69, 0.93, 1.16, 1.39, 1.85 or 2.31 g/L and urea concentrations were 5.22, 7.83, 10.43, 13.04, 15.65 or 20.87 g/L. Twenty members in each embryo group were submerged in a plastic petri dish (100 mm × 15 mm) with 40 mL of the appropriate solution. A dish with the individual solution was applied as the normal control. The experiment was performed at 21 ± 1°C with a 12-h light and 12-h dark photoperiod. The observed dead embryos and other remnants were immediately removed. The solution was refreshed every 24 h. After 96-h exposure, the experiment was terminated. All living embryos were fixed in 10% formalin solution. Developmental stages and malformations of embryos were examined under a stereomicroscope (SZ40 Olympus). The median lethal concentration (LC_{50})

value was determined from mortality, and the median effective concentration (EC_{50}) value was calculated from the abnormal embryos. The teratogenic index value was derived from LC_{50} divided by EC_{50} at 96 h (Fabro et al., 1982)

Statistical Analysis

The obtained data were statistically analysed with IBM SPSS Statistics version 20.0 for Mac OS X (IBM Corp., Armonk, NY, USA). Probit analysis was applied for calculating the lethal concentration (LC_x) and the effective concentration (EC_x) values that induced malformation. One-way analysis of variance (ANOVA) with post-hoc Tukey's honest significant difference (HSD) test was used to analyse the developmental stage.

RESULTS

Mortality

No mortality was observed in the control group. As shown in Table 1, exposure of *H. rugulosus* embryos to either ammonium sulphate or urea fertilisers produced lethal effects, but to varying degrees. The LC_x values of urea-exposed embryos were significantly greater compared to ammonium sulphate exposure at both the gastrula and neurula stages. For the same fertiliser, neurulae showed a markedly higher LC_{50} value compared to gastrulae.

Teratogenicity

There were no abnormal features observed in the control group. The calculated EC_x for teratogenesis and teratogenic index (T.I.) values for the two fertilisers are shown in Table 2. In addition to the LC values, the EC_{50} values for ammonium sulphate and urea fertilisers on *H. rugulosus* embryonic stages increased with higher effective concentrations. Moreover, the urea-fertiliser-treated embryos presented remarkably higher EC values at both embryonic stages compared to ammonium sulphate fertiliser treatment. Furthermore, gastrulae exposed to ammonium sulphate fertiliser demonstrated lower EC values compared to neurulae for all effective concentrations. Similarly, gastrulae exposed to urea fertiliser also

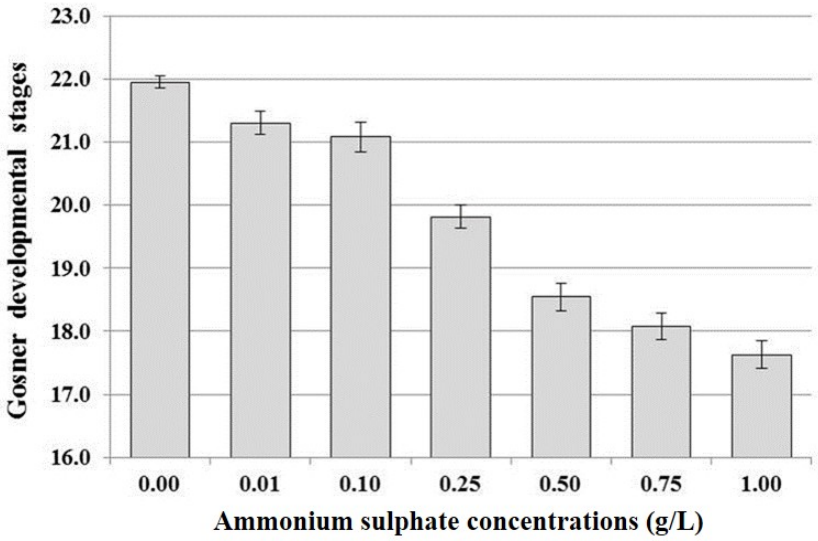
TABLE 1. Lethal concentrations of ammonium sulphate and urea fertilisers exposed to gastrulae and neurulae of *H. rugulosus* for 96 hours (values with 95% confidence limits)

Lethal concentration degree	Lethal concentrations (g/L)			
	ammonium sulphate fertiliser		urea fertiliser	
	Gastrula	Neurula	Gastrula	Neurula
LC_{10}	0.18 (0.02-2.95)	0.69 (0.53-0.82)	4.00 (2.86-4.94)	9.52 (8.06-10.60)
LC_{50}	0.93 (0.80-1.11)	1.38 (1.29-1.49)	10.43 (9.63-11.32)	14.86 (14.00-15.81)
LC_{90}	1.67 (1.42-2.08)	2.07 (1.91-2.28)	16.87 (15.57-18.54)	20.21 (18.87-22.10)

TABLE 2. Effective concentrations of ammonium sulphate and urea fertilisers exposed to gastrulae and neurulae of *H. rugulosus* for 96 hours (values with 95% confidence limits)

Effective concentration degree	Effective concentrations (g/L)			
	ammonium sulphate fertiliser		urea fertiliser	
	Gastrula	Neurula	Gastrula	Neurula
EC_{10}	0.10 (0.00-0.19)	0.73 (0.5-0.89)	1.54 (0.55-2.35)	10.92 (8.80-12.53)
EC_{50}	0.64 (0.80-1.11)	1.58 (1.37-1.93)	6.95 (6.10-8.01)	17.91 (15.73-22.46)
EC_{90}	1.18 (1.00-1.47)	2.42 (2.04-3.18)	12.36 (10.87-14.46)	24.91 (20.94-34.11)
T.I.	1.45	0.87	1.50	0.83

FIGURE 1. Gosner developmental stages of the average gastrulae treated with gradient concentrations of ammonium sulphate fertiliser for 96 hours.



presented lower EC values compared to neurulae. The T.I. values of gastrulae were higher than those of neurulae if treated with the same kind of fertiliser. There were no significant differences in T.I. values for the same embryonic stage between the two fertilisers.

FIGURE 2. Gosner developmental stages of the average neurulae treated with gradient concentrations of ammonium sulphate fertiliser for 96 hours.

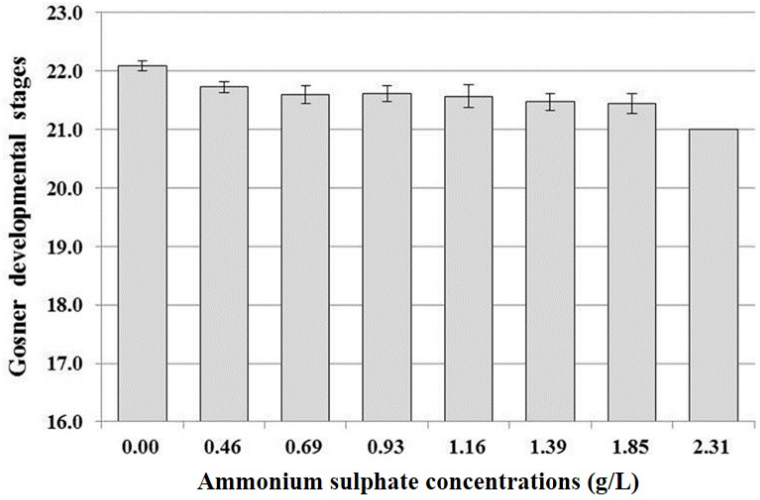
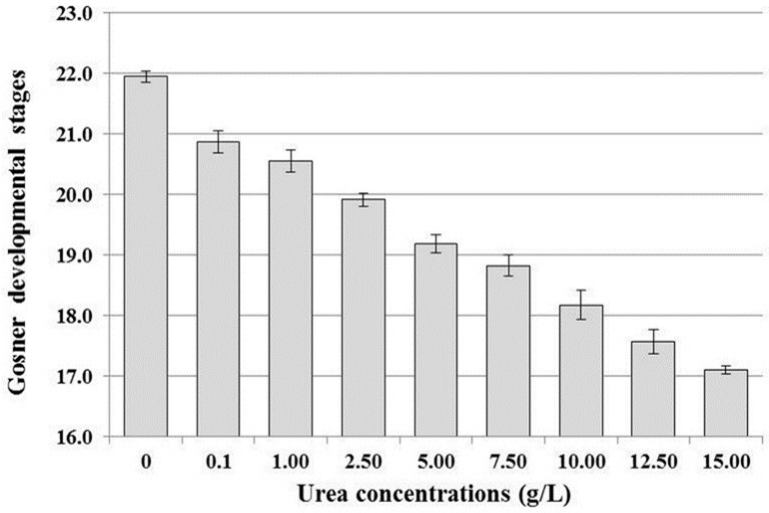


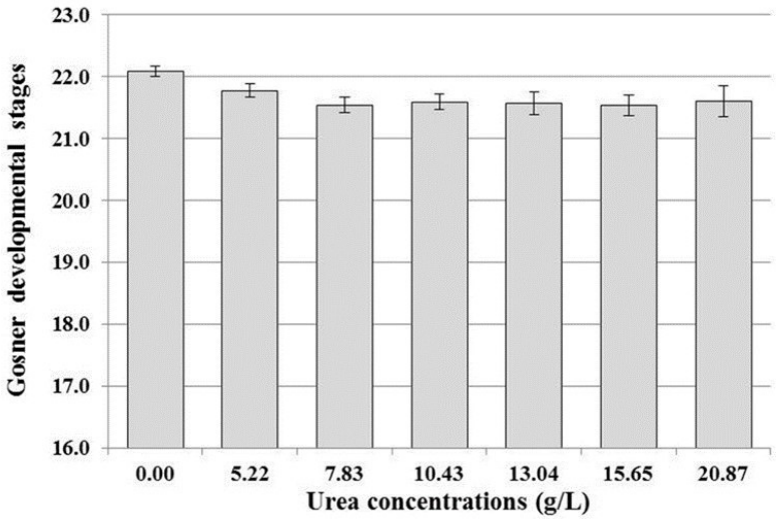
FIGURE 3. Gosner developmental stages of the average gastrulae treated with gradient concentrations of urea fertiliser for 96 hours.



Developmental Effects

At the end of the experiment, the average developmental stages of gastrulae and neurulae in the control groups were 21.95 ± 0.10 and 22.08 ± 0.08, respectively (Figs. 1-4). In fertiliser-treated groups, the gastrulae and

FIGURE 4. Gosner developmental stages of the average neurulae treated with gradient concentrations of urea fertiliser for 96 hours.

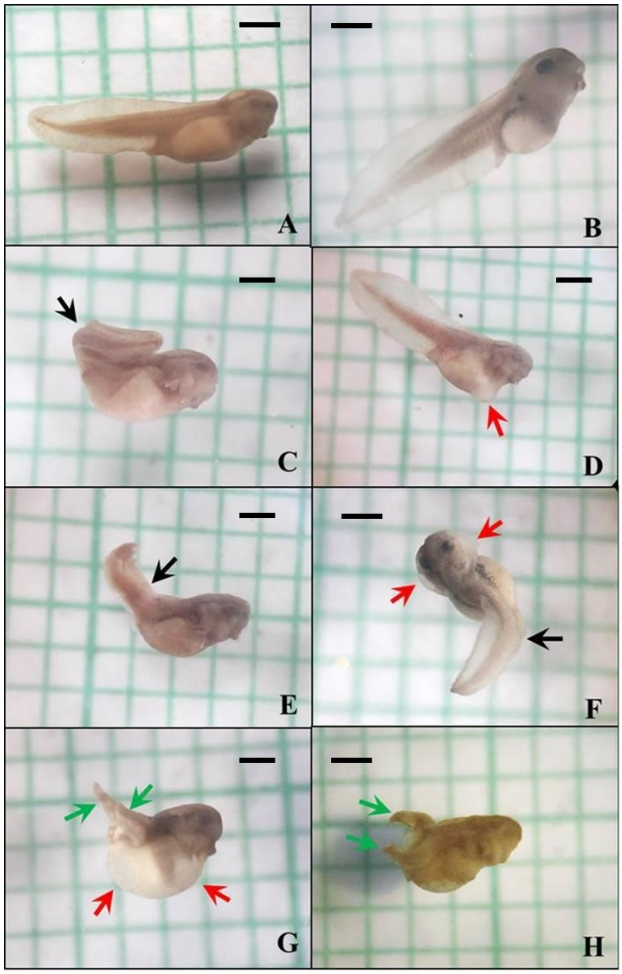


neurulae development was notably slower as the fertiliser concentration increased (Figs. 1-4). However, fertiliser-exposed gastrulae (Figs. 1, 3) tended to exhibit a greater reduction in development compared to neurulae (Figs. 2, 4). The average developmental stage of 96-h gastrulae and neurulae treated with ammonium sulphate were 17.63 ± 0.22 (at 1 g/L ammonium sulphate) and 21.00 ± 0.00 (at 2.31 g/L of ammonium sulphate), respectively (Figs. 1, 2). Meanwhile, the average developmental stages for 96-h gastrulae and neurulae treated with urea fertiliser were 17.10 ± 0.07 (at 15 g/L urea) and 21.6 ± 0.54 (at 20.87 g/L urea; Figs. 3, 4).

Abnormal Features

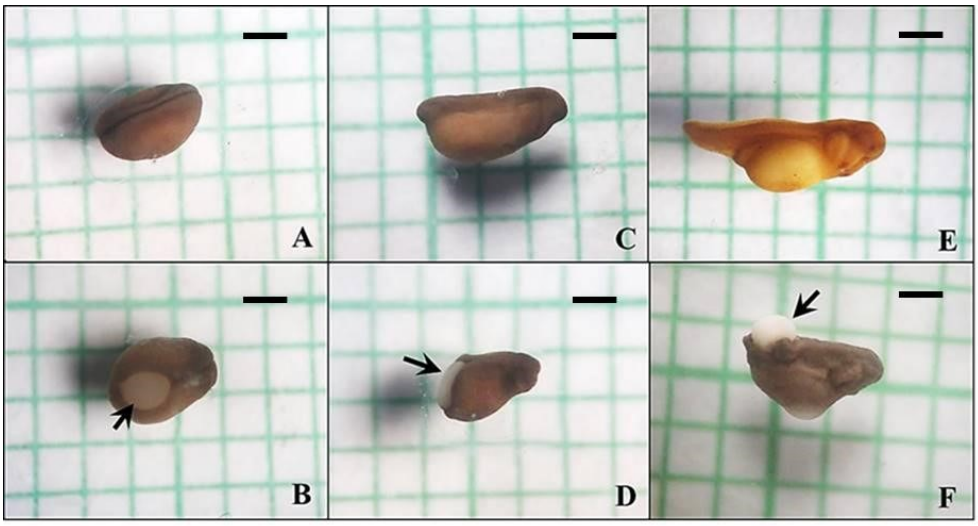
Control embryos did not exhibit any abnormal features (Figs. 5A, 5B). Ammonium sulphate and urea fertiliser exposure induced abnormality in both gastrulae and neurulae as follows: oedema, body and tail kinking and a multiple tail-like structure. Oedema (Figs. 5D, 5F, 5G) and kinking of the body and tail (Figs. 5C, 5E, 5F) were observed in gastrulae and neurulae, whereas the presence of the multiple tail-like structure (Figs. 5G, 5H) was recorded only in gastrulae exposed to these fertilisers. The multiple tail-like structure usually occurred with the presence of the yolk plug during development. In the control group, the yolk plug disappeared at Gosner stage 13 and the neural groove laid from the anterior to posterior ends of the dorsal side presented by Gosner stage 15 (Fig. 6A). In the urea-fertiliser-treated group, embryos in Gosner stage 15 still possessed the yolk plug on the posterior end and thus had a shorter neural groove (Fig. 6B). Additionally, normal embryos in Gosner stage 17 began to develop a distinct head on the anterior end and the tail bud on the posterior end (Fig. 6C). Comparatively, the embryo exposed to urea fertiliser showed normal development of the anterior end, but the neural groove and tail development on the posterior end were

FIGURE 5. Features of *H. rugulosus* embryos (20X) after 96 hours of the experiment
A: Embryo in Gosner stage 21 of control group (started from Gosner stage 10)
B: Embryo in Gosner stage 23 of control group (started from Gosner stage 13)
C: Embryo in Gosner stage 21 of urea exposed group (started from Gosner stage 10)
D: Embryo in Gosner stage 22 of urea exposed group (started from Gosner stage 13)
E: Embryo in Gosner stage 21 of ammonium sulphate exposed group (started from Gosner stage 10)
F: Embryo in Gosner stage 22 of ammonium sulphate exposed group (started from Gosner stage 13)
G: Embryo in Gosner stage 21 of urea exposed group (started Gosner stage 10)
H: Embryo in Gosner stage 20 of ammonium sulphate exposed group (started from Gosner stage 10)
Black arrow = kinked tail, Red arrow = oedema, Green arrow = multiple tail-like structure, Scale bar = 1 mm



restricted (Fig. 6D). At Gosner stage 19, control embryos exhibited a long tail, normal heart and head formation (Fig. 6E). However, the urea-fertiliser-treated embryos showed normal head development, but the large yolk plug at the posterior region was surrounded by multiple buds of tail-like structure (Fig. 6F).

FIGURE 6. Features of *H. rugulosus* embryos (20X) during the 96 hours of the experiment (started from Gosner stage 10)
A: Embryo in Gosner stage 15 of control group
B: Embryo in Gosner stage 15 of urea exposed group
C: Embryo in Gosner stage 17 of control group
D: Embryo in Gosner stage 17 of urea exposed group
E: Embryo in Gosner stage 19 of control group
F: Embryo in Gosner stage 19 of urea exposed group
Black arrow = yolk plug, Scale bar = 1 mm



DISCUSSION

Mortality

The present study is the first report to reveal the lethal toxicity of ammonium sulphate and urea fertilisers on embryonic stages of *H. rugulosus,* a common Asian amphibian. Until now, the lethal toxicity of these two fertilisers was mainly focused on fish and snails (Ofojekwu et al., 2008; Capkin et al., 2010; Essien-ibok et al., 2015; Saxzena et al., 2016). However, the 96-h LC_{50} values for ammonium sulphate and urea fertilisers in amphibians in this study were quite similar to the values in previous studies. For example, the 96-h LC_{50} for ammonium sulphate fertiliser in the juvenile rainbow trout (*Oncorhynchus mykiss*) and the striped dwarf catfish (*Mystus vittatus*) are 0.15 and 0.30 g/L, respectively (Capkin et al., 2010; Saxzena et al., 2016). The 96-h LC_{50} values of urea fertiliser in redbelly tilapia (*Tilapia zilli*) fingerings and the African catfish (*Heterobranchus bidorsalis*) are 15.85 g/L and 17.84 g/L, respectively (Ofojekwu et al., 2008; Essien-ibok et al., 2015).

According to our LC_{50} values, ammonium sulphate fertiliser exhibited a higher lethal toxicity to *H. rugulosus* compared to urea fertiliser. These data are consistent with an experiment in snails. Tchounwou et al. (1991) presented the 24-h LC_{50} values of ammonium sulphate and urea fertilisers in

the freshwater air-breathing snail *Helisoma trivolvis* (393 mg/L versus 18.26 g/L) and the ghost rams-horn snail *Biomphalaria havanensis* (526 mg/L versus 24.50 g/L). Moreover, frog embryos in the neurula stage (later stage) were more tolerant to the two nitrogen fertilisers than gastrula embryos (earlier stage). Gastrulation is the critical stage of embryonic development and consists of myriad processes, including cell displacement, cellular adhesiveness and cellular interactions (Monroy and Moscona, 1979; Edelman, 1984). It is possible that ammonium sulphate and urea might interfere with these processes and cause the gastrula to be more sensitive than the neurula. Tchounwou et al. (1991) reported that the LC_{50} of ammonium sulphate and urea in juveniles and adults of *Helisoma trivolvis* exposed to fertilisers for 24 h are 0.56 (juveniles) to 0.70 g/L (adult) and 14.2 (juveniles) to 30.1 (adult) g/L, respectively. Urea is less toxic than ammonia and ammonium (Wright, 1995). Therefore, the ammonium ion released from ammonium sulphate could underlie the greater toxicity to *H. rugulosus* embryos compared to urea. Obviously, the difference in toxic degree of these two fertilisers might be a consequence of exposure time, developmental stage of embryo, life stage, life history, and animal species.

Teratogenicity

Based on EC values, both ammonium sulphate and urea fertilisers produced teratogenic effects to *H. rugulosus* gastrulae and neurulae. Urea, with higher EC values, was less teratogenic to frog embryos compared to ammonium sulphate. Moreover, gastrulae (with a lower EC) were more vulnerable to malformation induced by these two fertilisers compared to neurulae.

There is limited information about EC_{50} values of fertilisers and growth enhancers in amphibians. Schuytema and Nebeker (1999) reported that the EC_{50} value of ammonium nitrate in the African clawed frog *Xenopus laevis* embryos is 0.149 g/L. The 96-h EC_{50} value of gibberellic acid in the *X. laevis* embryos is 0.659 g/L (Boğa et al., 2008). Therefore, the 96-h EC_{50} values of ammonium sulphate and urea fertilisers in this study represented the low range of teratogenic effects to frog embryos when compared to other agrochemicals.

In terms of T.I. values, gastrulae were more sensitive and exhibited a greater potential for fertiliser-induced teratogenicity compared to neurulae. Meanwhile, both fertilisers exhibited a similar potential to induce abnormality in the same embryonic stage. However, the teratogenic effect of fertilisers has been poorly characterised due to the limited number of studies on this issue.

Developmental Effects

In the groups of treatment, exposure to ammonium sulphate and urea solutions significantly suppressed the development of *H. rugulosus* gastrulae and neurulae. However, gastrulae tended to present more adverse effects after treatment with either fertiliser compared to neurulae; these data are consistent with their different sensitivity. According to the average development of embryos exposed to these fertilisers, it is obvious that ammonium sulphate reduced embryonic development more than urea.

The developmental effects of ammonium sulphate and urea fertilisers were previously investigated at the cellular and organ levels of histopathological damage in various kinds of animals. Ram and Sathyanesan (1987) noted that 0.5 g/L ammonium sulphate induces hepatocyte necrosis in the spotted snakehead (*Channa punctatus*). Ammonium sulphate fertiliser affects the epidermis of *Heteropneustes fossilis*; it causes massive

destruction, including mucous cell irritation, necrosis sloughing, and shrinkage of epithelial cells (Banerjee and Paul, 1993). Spillage of urea fertiliser acutely poisons silver gulls (*Larus novaehollandiae*) and causes congestion of visceral organs and brain (Raidal and Jaensch, 2006). Gupta (2016) stated that *H. fossilis* exposed to urea have serious hyperplasia of gill epithelium, fusion and swollen gill lamellae with myriad mucous cells and dislocation of epithelial cells. However, there are no reports that mention the effect of these two fertilisers on amphibian embryonic development. Therefore, the effects of ammonium sulphate and urea fertilisers on the development of *H. rugulosus* embryonic stages were revealed for the first time in this study.

Besides the toxic effects of the nitrogen fertilisers, the ammonium ion (NH_4^+) could interfere with frog embryonic development. The ammonium ion is typically generated by dissolving ammonia in water or by nitrogen fertiliser breakdown. Ammonium produces toxic effects to gill epithelial cells and embryos in various kinds of animals. Devaraj et al. (2014) reported swelling and hyperplasia of gill epithelium in the common carp (*Cyprinus carpio*) treated with ammonia. Seventy-five μM ammonia in media significantly reduces the number of blastocyst cells in mouse embryo culture (Gardner and Lane, 1993). Additionally, 132 μM ammonium chloride significantly decreases the rate of cell division in cleavage and percentage of zygotes developing to blastocysts in ovine embryos (Golchin et al., 2015). Furthermore, ammonium ions reportedly act as a growth inhibitor for various cell lines (Glacken et al., 1986; Miller et al., 1988; Ozturk et al., 1992; Reuveny et al., 1986) and have inhibitory effects on glutamate dehydrogenase. The latter effect causes a shortage of alpha ketoglutarate in tricarboxylic acid (TCA) cycle (Glacken et al., 1988). As a consequence of disturbing the TCA cycle, the exposed cells would have a shortage of energy (ATP) for cellular activities, including the movement of spindle fiber during cytokinesis and the Na^+/K^+ ATPase pump, both of which will interrupt embryonic development.

No organ development was observed in the gastrulae, and thus cellular toxicity was hypothesised as the main reason of the delayed embryonic development. In gastrulation, three different cellular movements are described (epiboly, invagination, and involution) that result in ectoderm, mesoderm, and endoderm formation. Later, neurulation begins and allows neural tissue development as initiated by chordamesoderm. During gastrulation, cell movement and differentiation occur in sequences. Initially, cell division produces a movement force for epiboly, invagination, and involution. As the group of cells around the lower margin of grey crescent sinks into the embryo to create a small groove, cells on the upper side of the groove move inward along the inner surface of the animal's hemisphere (involution). Later, these involuted cells differentiate into various types of mesoderm and also initiate the differentiation of outer cells (Gilbert, 2006). If any factors cause cellular damage or disturb any steps of cell division/cell movement, the subsequent development would be delayed. If ammonium sulphate and urea fertilisers damage the lip of the blastopore, the movement force would disappear. Subsequently, the groups of marginal cells that move inward more slowly or later than those of a normal embryo. The late involution would also delay the neural formation, head development and the subsequent processes.

Abnormal Features

In the current study, exposure to ammonium sulphate and urea fertilisers induced various abnormalities (oedema, kinking and a multiple tail-like structure) in frog embryos. Oedema and body and tail kinking were observed in previous studies (Lien et al., 1997; Greulich and Pflugmacher, 2003; Lin et al., 2007). For example, Lin et al. (2007) mentioned pericardiac and abdominal oedema in zebrafish (*Danio rerio*) embryos treated with carbaryl. Additionally, Lien et al. (1997) reported kinked tails in catfish larva induced by malathion insecticide. Body oedema, tail kinking and craniofacial abnormalities were reported in *Xenopus laevis* embryos exposed to sodium chromate (Greulich and Pflugmacher, 2003). Therefore, oedema and kinking of any structures found in frog and fish embryos are assumed to occur without specificity to chemical agents.

Oedema generally occurs due to chemical agents that interfere with development or trigger damage to the pronephros and lead to kidney dysfunction (Hill et al., 2004). Oedema can also be stimulated by exposure to ammonia, which increases the level of free radicals within astrocytes and makes them swollen (Norenberg et al., 2009). In the case of kinking, there are several documents to support that the availability of ammonium ion can reduce the developmental rate in blastocysts and alter gene expression patterns (Lane and Gardner, 2003; Golchin et al., 2015) to promote tail and neural tube kinking.

The present study provides the first evidence of a multiple tail-like structure in frog embryos and also describes the teratogenic effect of agrochemicals on tail development. On the contrary, multiple limbs and other limb malformations (absence or short limbs) were previously observed (Sessions and Ruth, 1990; Tabin, 1991; Stratford et al., 1996, 1999; Johnson et al., 1999; Sessions et al., 1999; Maden and Hind, 2003) and induced by several factors, parasitic infection and/or chemical contamination. For instance, the presence of multiple hind limb buds in amphibian larvae is initiated by trematode flatworm *Ribeiroia* sp. infection (Sessions and Ruth, 1990; Johnson et al., 1999; Sessions et al., 1999). This effect appears to be specific for the host species and parasite. Limb development, regeneration and deformity in amphibians are regulated by retinoic acid (Maden and Hind, 2003) via control of *Shh*, *fgf4*, *bmp2*, and *Hox* gene expression (Tabin, 1991; Stratford et al., 1996, 1999). Additionally, newly metamorphosed *X. laevis* exposed to contaminated water and sediments since the embryonic stage exhibit high rates of impaired limbs (Fort et al., 1999). Notably, multiple limbs were induced by several specific factors that are available in limited areas. In contrast, the multiple tail-like structure was induced by fertilisers that are commonly used worldwide, especially in agricultural fields.

Moreover, the mechanism of the multiple tail-like structure is different from that of multiple limbs: it is a supposed consequence of a cellular effect rather than a genetic effect. If the abnormality was the result of changes in gene expression that control tail development, both gastrulae and neurulae would exhibit the defect. However, only the gastrulae showed this effect. Hence, this abnormality between the two embryonic stages is considered to result from impairment and toxicity effects of fertilisers at cellular levels. The multiple tail-like structure was apparent with the presence of large yolk plug in gastrulae. It indicated that the exposure to ammonium sulphate and

urea, including their ammonium ions, may affect cell movement in gastrulation.

In gastrulation, the yolk plug is the group of remaining cells at the vegetal pole and finally folds internally into the embryo and becomes the endoderm. The tail bud is created by the interaction between caudal notochord and neural plate (Beck and Slack, 1998). Therefore, the observed multiple buds of tail-like structure located to the side of the remaining yolk plug in the gastrula-stage embryos indicates that the caudal notochord was also divided into multiples and located underneath the tail buds. As the notochord is differentiated from chorda-mesoderm cells by the involution of marginal cells over the dorsal lip of blastopore (Parson et al., 2002), the presence of multiple buds of tail-like structure suggests delayed involution during gastrulation. The abnormality in late involution was confirmed by the presence of the multiple tail-like structure in gastrulae- and not neurulae-treated with fertilisers.

However, the presence of a large yolk plug was assumed to be the main cause of the multiple tail-like structure. During gastrulation, cells at the animal pole expand and migrate to the vegetal pole in order to cover the entire embryo (epiboly), except the small yolk plug (Gilbert, 2006). Exposure to ammonium sulphate or urea fertilisers may affect the outer cells by delaying or preventing epiboly. The reduced movement in the epiboly would lead to the presence of the large yolk plug. While the epiboly continues, the earlier involuted inner cells that are less affected by the fertilisers may begin to induce the outer cells. This event would lead to differentiation and formation of neural plate. Since the yolk plug enlarges and epiboly is delayed, the inner cells along the sides of yolk plug start to create a tail bud from notochord and neural plate by themselves. Thus, multiple buds of tail-like structure are generated along with the multiple branches of the caudal notochord.

The current results are consistent with Zhang et al. (2010), who reported that ethanol exposure during gastrulation delays the epiboly and convergence extension and results in split axes in zebrafish. The split axes in ethanol-exposed zebrafish embryos are observed during the 64-cell to the germ ring stages. Zhang et al. (2010) also investigated various genetic markers for axis development, but they claimed that the abnormal cell movement during gastrulation is the key to induce splitting of the axis in zebrafish embryos.

Head development of fertiliser-treated embryos (Gosner stages 15-19) was normal when compared to control group. These data suggest that head formation in the early gastrula embryos (Gosner stage 10) was not affected by nitrogen fertiliser exposure. Even though the present study did not indicate the exact mechanism for these fertiliser-induced abnormalities, the negative effects of both fertilisers on the embryonic development were certainly documented. Moreover, the toxicity of ammonium sulphate and urea fertilisers in amphibian embryos tended to affect the cell movement similar to the other vertebrates. The results from this study may raise awareness to the effects of ammonium sulphate and urea fertilisers to the embryos of aquatic animals. This knowledge will hopefully lead to the careful application of agrochemicals in farming.

CONCLUSION

In conclusion, the present study is the first report of a multiple tail-like abnormality

in amphibian embryos exposed to nitrogen fertilisers. Ammonium sulphate and urea fertilisers severely affected *H. rugulosus* embryos, especially the gastrulae. This study suggests that the abnormality occurred due to interference with cell movements during gastrulation. Therefore, the two fertilisers may induce abnormal development in other amphibians and vertebrates by the same mechanism. The present study will hopefully raise awareness of ammonium sulphate and urea fertiliser contamination to the environment.

ACKNOWLEDGEMENTS

We would like to thank Emma Taylor for English proofreading. We also would like to express our gratitude to the Research Professional Development Project and the Graduate School, Chiang Mai University, for financial supports.

LITERATURE CITED

Banerjee, T.K. and Paul, V.I. 1993. Estimation of acute toxicity of ammonium sulfate to the freshwater catfish, *Heteropneustes fossilis*. II. A histopathological analysis of the epidermis. Biomedical and Environmental Sciences, 6: 45-58.

Beck, C.W. and Slack, J.M.W. 1998. Analysis of the developing *Xenopus* tail bud reveals separate phases of gene expression during determination and outgrowth. Mechanisms of Development, 72(1): 41-52.

Boğa, A., Bİnokay, S. and Sertdemİr, Y. 2008. The toxicity and teratogenicity of gibberellic acid (GA 3) based on the frog embryo teratogenesis assay-*Xenopus* (FETAX). In Vitro Cellular and Developmental Biology, 33: 181-188.

Capkin, E., Kayis, S., Boran, H. and Altinok, I. 2010. Acute toxicity of some agriculture fertilizers to rainbow trout. Turkish Journal of Fisheries and Aquatic Sciences, 25: 19-25.

Devaraj, S., Arulprakasam, C., Kandhan, A.P., Neelamegam, K. and Kalaiselvan, R. 2014. Toxicological effects of ammonia on gills of *Cyprinus carpio* var. *communis* (Linn.). Journal of Coastal Life Medicine, 2(2): 94-98.

Edelman, G.M. 1984. Cell adhesion and morphogenesis: the regulator hypothesis. Proceedings of the National Academy of Sciences of the United States of America, 81: 1460-1464.

Essien-ibok, M.A., Asuquo, I.E. and Ekpo, I.E. 2015. The assessment of acute toxicity of urea fertilizer against *Heterobranchus bidorsalis* fingerlings. Global Journal of Fisheries and Aquaculture, 2(5): 169-176.

Fabro, S., Shull, G. and Brown, N.A. 1982. The relative teratogenic index and teratogenic potency: proposed components of developmental toxicity risk assessment. Teratogenesis, Carcinogenesis, and Mutagenesis, 2(1): 61-76.

Food and Agriculture Organization of the United Nations (FAO). 2016. FAOSTAT Database. Retrieved Mar 16, 2016, from http://faostat.fao.org/site/424/DesktopDefault.aspx? PageID=424.

Fort, D.J., Propst, T.L., Stover, E.L., Helgen, J.C., Levey, R.B., Gallagher, K. and Burkhart, J.G. 1999. Effects of pond water, sediment, and sediment extracts from Minnesota and Vermont, USA, on early development and metamorphosis of *Xenopus*. Environmental Toxicology and Chemistry, 18(10): 2305-2315.

Fryday, S. and Thompson, H. 2012. Toxicity of Pesticides to Aquatic and Terrestrial Life Stages of Amphibians and Occurrence, Habitat Use and Exposure of Amphibian Species in Agricultural Environments. Supporting Publications, Food and Environment research Agency, York, 348 pp.

Gardner, D.K. and Lane, M. 1993. Amino acids and ammonium regulate mouse embryo development in culture. Biology of Reproduction, 48(2): 377-385.

Gilbert, S.F. 2006. Developmental Biology, 6th Ed. Sinauer Associates, Sunderland, 709 pp.

Glacken, M.W., Adema, E. and Sinskey, A.J. 1988. Mathematical description of hybridoma culture kinetics: I. Initial metabolic rates. Biotechnology and Bioengineering, 32: 491-506.

Glacken, M.W., Fleischaker, R.J. and Sinskey, A.J. 1986. Reduction of waste product excretion via nutrient control: possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnology and Bioengineering, 28: 1376-1389.

Golchin, A., Asadpour, R., Roshangar, L. and Jafari-jozani, R. 2015. The effect of ammonium chloride concentration in in vitro maturation culture on

ovine embryo development. Journal of Reproduction and Infertility, 17(3): 144-150.

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

Greulich, K. and Pflugmacher, S. 2003. Differences in susceptibility of various life stages of amphibians to pesticide exposure. Aquatic Toxicology, 65(3): 329-336.

Gupta, T. 2016. Effect of short term exposure to urea on catfish (*Heteropneustes fossilis*) a light microscopic study of the gills. European Journal of Pharmaceutical and Medical Research, 3(1): 161–163.

Hill, A.J., Bello, S.M., Prasch, A.L., Peterson, R.E. and Heideman, W. 2004. Water permeability and TCDD-induced edema in zebrafish early-life stages. Toxicological Sciences, 78(1): 78-87.

Holtfreter, J. 1931. Uber die aufzucht isolierter teile des amphibian keimes II. Archiv für Entwicklungsmechanik der Organismen, 124: 404–465.

Houlahan, J.E., Findlay, C.S., Schmidt, B.R., Meyer, A.H. and Kuzmin, S.L. 2000. Quantitative evidence for global amphibian population declines. Nature, 404: 752-755.

Husk, B.R., Anderson, B.C., Whalen, J.K. and Sanchez, J.S. 2017. Reducing nitrogen contamination from agricultural subsurface drainage with denitrification bioreactors and controlled drainage. Biosystems Engineering, 153: 52-62.

Johnson, P.T.J., Lunde, K.B., Ritchie, E.G. and Launer, A.E. 1999. The effect of trematode infection on amphibian limb development and survivorship. Science, 284: 802–804.

Lane, M. and Gardner, D.K. 2003. Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. Biology of Reproduction, 69(4): 1109-1117.

Lien, N.T., Adriaens, D. and Janssen, C.R. 1997. Morphological abnormalities in African catfish (*Clarias gariepinus*) larvae exposed to malathion. Chemosphere, 35: 1475–1486.

Lin, C.C., Hui, M.N.Y. and Cheng, S.H. 2007. Toxicity and cardiac effects of carbaryl in early developing zebrafish (*Danio rerio*) embryos. Toxicology and Applied Pharmacology, 222(2): 159–168.

Marco, A., Cash, D., Belden, K. and Blaustein, A.R. 2001. Sensitivity to urea fertilization in three amphibian species. Archives of Environmental Contamination and Toxicology, 40: 406-409.

Maden, M. and Hind, M. 2003. Retinoic acid, a regeneration-inducing molecule. Developmental Dynamics, 226(2): 237-244.

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

Miller, W.M., Wilke, C.R. and Blanch, H.W. 1988. Transient responses of hybridoma cells to lactate and ammonia pulse and step changes in continuous culture. Bioprocess Engineering, 3: 113-122.

Monroy, A. and Moscona, A.A. 1979. Introductory Concepts in Developmental Biology. University of Chicago Press, Chicago, 252 pp.

Norenberg, M.D., Rama-Rao, K.V. and Jayakumar, A.R. 2009. Signaling factors in the mechanism of ammonia neurotoxicity. Metabolic Brain Disease, 24: 103-117.

Ofojekwu, P.C., Nwani, C.D. and Ihere, R.E. 2008. Acute toxicity of urea fertilizer to *Tilapia zilli* fingerlings. Bio-Research, 6(1): 298-300.

Ozturk, S.S., Riley, M.R. and Palsson, B.O. 1992. Effects of ammonia and lactate on hybridoma growth, metabolism, and antibody production. Biotechnology and Bioengineering, 39: 418-431.

Parsons, M.J., Pollard, S.M., Feldman, B., Coutinho, P., Hirst, E.M. and Stemple, D.L. 2002. Zebrafish mutants identify an essential role for laminins in notochord formation. Development, 129(13): 3137-3146.

Raidal, S.R. and Jaensch, S.M. 2006. Acute poisoning of silver gulls (*Larus novaehollandiae*) following urea fertilizer spillage. Avian Pathology, 35(1): 38-41.

Ram, R.N. and Sathyanesan, A.G. 1987. Histopathological changes in liver and thyroid of the teleost fish, *Channa punctatus* (Bloch), in response to ammonium sulfate fertilizer treatment. Ecotoxicology and Environmental Safety, 13(2): 185-190.

Reuveny, S., Velez, D., Macmillan, J.D. and Miller, L. 1986. Factors affecting cell growth and monoclonal antibody production in stirred reactors. Journal of Immunological Methods, 86: 53-59.

Saxena, P., Kumar, P., Nagpure, N.S., Kumar, D. and Mathur, P.K. 2016. Evaluation of lethal concentration of ammonium sulphate to freshwater fish *Mystus vitatus* (BLOCH). Journal of Biological Sciences, 2(3): 1-5.

Schuytema, G.S. and Nebeker, A.V. 1999. Comparative toxicity of ammonium and nitrate

compounds to Pacific treefrog and African clawed frog tadpoles. Environmental Toxicology and Chemistry, 18(10): 2251-2257.

Sessions, S.K., Franssen, R.A. and Horner, V.L. 1999. Morphological clues from multilegged frogs: are retinoids to blame?. Science, 284: 800-802.

Sessions, S.K. and Ruth, S.B. 1990. Explanation of naturally occurring supernumerary limbs in amphibians. Journal of Experimental Zoology, 254: 38-47.

Stratford, T., Horton, C. and Maden, M. 1996. Retinoic acid is required for the initiation of outgrowth in the chick limb bud. Current Biology, 6: 1124-1133.

Stratford, T., Logan, C., Zile, M. and Maden, M. 1999. Abnormal anteroposterior and dorsoventral patterning of the limb bud in the absence of retinoids. Mechanisms of Development, 81: 115-125.

Tabin, C.J. 1991. Retinoids, homeoboxes, and growth factors: Toward molecular models for limb development. Cell, 66: 199-217.

Tchounwou, P.B., Englande, A.J. Jr. and Malek, E.A. 1991. Toxicity evaluation of ammonium sulphate and urea to three developmental stages of freshwater snails. Archives of Environmental Contamination and Toxicology, 21: 359-364.

Wijer, P., Watt, P.J. and Oldham, R.S. 2003. Amphibian decline and aquatic pollution: effects of nitrogenous fertiliser on survival and development of larvae of the frog *Rana temporaria*. Applied Herpetology, 1: 3-12.

Wright, P.A. 1995. Review nitrogen excretion: three end products, many physiological roles. The Journal of Experimental Biology, 198: 273-281.

Zhang, Y., Shao, M., Wang, L., Liu, Z., Gao, M., Liu, C. and Zhang, H. 2010. Ethanol exposure affects cell movement during gastrulation and induces split axes in zebrafish embryos. International Journal of Developmental Neuroscience, 28(4): 283-288.