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Full Paper

Effects of atrazine herbicide on metamorphosis and gonadal development of *Hoplobatrachus rugulosus*

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Abstract: Due to climate fluctuation, the major agricultural production of Thailand has shifted from rice to include many other crops with consequent increase in the use of a wider variety of herbicides. The demasculinisation of amphibians caused by atrazine has raised concern about population decline of the animals in this class. Although atrazine is commonly used in Thailand, little and controversial information exists regarding the impact of this herbicide on East-Asian bullfrog (*Hoplobatrachus rugulosus*), a native frog in Thailand. This research project is therefore designed to evaluate the toxicity of atrazine on the larvae of *H. rugulosus* by focusing on gonadal and metamorphosis impairments. The 14-day old larvae were exposed to atrazine herbicide at concentrations of 0.25, 2.50 and 25.0 ppb for 40 days compared to the untreated control group. The following data were investigated: survival, ratio of body length, tail length, appearance of limbs and gonadal morphology. It was found that the herbicide significantly delayed growth and metamorphosis of the larvae ($P < 0.05$). A higher ratio of female to male was also markedly observed in groups treated with atrazine herbicide. The development of gonadal intersex (ovotestes) was also induced by atrazine exposure. This research may act as a messenger to raise awareness about the risk of atrazine herbicide to our native frog species.

Keywords: herbicide, atrazine, *Hoplobatrachus rugulosus*, metamorphosis, gonad, demasculinisation

Maejo Int. J. Sci. Technol. **2013**, *7*(Special Issue), 60-71

INTRODUCTION

The population decline and feminisation of amphibians have recently become a topic of interest as a result of an increasing role of agricultural chemicals used in farming. Atrazine, a widely used herbicide, is the prime suspect in the feminisation of amphibians. This herbicide is applied to croplands and rice fields to exterminate broadleaf and grassy weeds [1]. Although atrazine has been banned in the European Union [2], it is still used heavily in many parts of the world. Approximately 30,000 tons of atrazine were used in the United States in 1997 [3] and 192 tons were used in the Netherlands in 1985 [4]. The rate of atrazine usage was reported to be 5,000 tons per year in China [5] and 1,088-2,935 tons per years in Thailand during 1993-2000 [6]. From this information, the fact that the ecosystem has suffered as a consequence of atrazine contamination [7] is not at all surprising. The impact of atrazine on wildlife and human health has been documented [8-12]. Nevertheless, its toxicity on human health remains controversial [13]. On the contrary, obvious adverse effects of atrazine to amphibians have largely been known. Survival reduction of tadpoles and juvenile frogs in many areas of atrazine application has been observed [7, 14-15]. The LC_{50} of atrazine was reported as 100 ppm for *Xenopus laevis* [16], 47.6 ppm for *Rana pipiens* [17] and 26.5-48 ppm for *Bufo americanus* [17-18]. This direct effect of atrazine on amphibian survival rate has led to a population decline and has attracted global attention. However, the consequences of non-lethal effects of atrazine on amphibians can indirectly bring about striking impact on their population as well. It was shown by the study of Hayes *et al.* [19] that relatively small concentration of atrazine (0.1 ppb) could demasculinise *X. laevis* larvae and turn developing frogs into hermaphrodites. The xenoestrogenic effects of atrazine and the endocrine-disrupting effects have been proposed for this phenomenon [19-21].

Besides being an important member of the ecological system, amphibians are also economically important animals in Thailand. They are a very good source of protein for local people and frozen frog legs of farm-raised frogs are a part of the exports to many countries [22]. The effects of atrazine on *Hoplobatrachus rugulosus* were preliminarily studied by Katawutpoonphan [23]. Surprisingly, atrazine concentrations of up to 1,000 ppb did not produce the same adverse effects on *H. rugulosus* [23] as it does with the other amphibian species that appear to be very sensitive to atrazine exposure [19-21]. Due to the widespread use of atrazine in Thailand, the present study was thus conducted to investigate the effects of atrazine herbicide on the metamorphosis and gonadal development of *H. rugulosus*, a native frog of Thailand.

MATERIALS AND METHODS

Animals and Chemical

Experiments were performed with *H. rugulosus* larvae. Tadpoles at 12 days of age having a total length of 3.054±0.05 cm were used in this study. They were obtained from Faicome frog farm, Chaing Mai province. They were transferred to the aerated plastic containers containing dechlorinated tap water (2 litres) and allowed to acclimatise in the laboratory condition for 1 week. Twice daily, the tadpoles were fed with commercial tadpole diet and any accumulated residues were removed.

Atrazine (commercial grade) was purchased from Pazana Co., Thailand. The commercial product consisted of 80% atrazine formulated in powder form. The treatment solutions were prepared by dissolving atrazine (80% purity) in dechlorinated tap water and diluting to the required

Maejo Int. J. Sci. Technol. **2013**, *7*(Special Issue), 60-71

concentrations. The tested concentrations used in this study were based upon the concentrations of atrazine detected in agricultural areas in Thailand [24].

Experimental Design

Sixty randomly selected tadpoles were divided into 4 groups. The control group was kept in atrazine-free water and the three experimental groups were exposed to atrazine at the concentrations of 0.25, 2.50 and 25.0 ppb. Each treatment was conducted in triplicate. During 40 days of the experimental period, all animals were observed for their growth, development and survival. Dead larvae, if present, were counted and removed daily. The renewal of water and atrazine solution was conducted every 3 days and body lengths from snout to anus were recorded at that time. Metamorphosis was observed by means of tail resorption and fore-limb emergence. The numbers of tadpoles at the 4 stages of metamorphosis (tadpole with no limb (TN), tadpole with hind limbs (TH), tadpole with four limbs (TF) and tadpole with complete tail resorption (TC)) were recorded daily. At the end of the experiment, the survivors of each group were sampled for sex identification.

Sex Identification

The sex of the tadpole was initially assessed by an examination of the gross morphology of the gonad under a dissecting microscope following the criteria of Hayes [25], slightly modified. Thereafter, a histological examination was used to evaluate the accuracy of morphological identification. The gonad was carefully removed, preserved in 4% formalin and processed for paraffin histology. A 5-mm section tissue was then stained with hematoxylin and eosin (H&E) and the larval sex was again identified by histological appearance [25].

Statistical Analyses

All statistical analyses were managed with Statistical Package for Social Science (SPSS) software version 20.0 for Mac OSX (SPSS Inc., IBM). Values were analysed by one-way analysis of variance. If treatment effects were found ($p < 0.05$), means were separated by Dunnett's t-test.

RESULTS AND DISCUSSION

Mortality

Mortality of the tadpoles was observed in all atrazine-exposed groups. As shown in Figure 1, the survival of tadpoles decreased with increasing concentration of atrazine. A hundred per cent of mortality occurred in tadpoles exposed to atrazine at the concentration of 25 ppb while only low mortality was observed in the control group. The tadpoles in the 25-ppb group could survive for only about 20 days of the experimental period. Although nearly half of the tadpoles survived the atrazine concentration of 2.5 ppb, survivors from this group had a smaller size and asymmetrical limbs (Figure 2). However, the mortality of tadpoles in the 0.25 ppb group was not significantly different from that of control.

The lethal concentrations obtained from our study are in contradiction with the studies of Katawutpoonphan [23], who reported that the increase of mortality of *H. rugulosus* embryo was not observed with exposure to pure (98%) atrazine at concentrations up to 1,000 ppb. The toxicity of pesticides to aquatic organisms appears to be affected by age, size and the formulation of chemicals [26-27]. For many amphibian species, the metamorphosis period seems to be a highly vulnerable stage [28-29]. The embryo at the mid-blastula stage of *H. rugulosus* used by Katawutpoonphan [23] might be less sensitive to atrazine than the older larvae used in our study. In addition to the age

Maejo Int. J. Sci. Technol. **2013**, *7*(Special Issue), 60-71

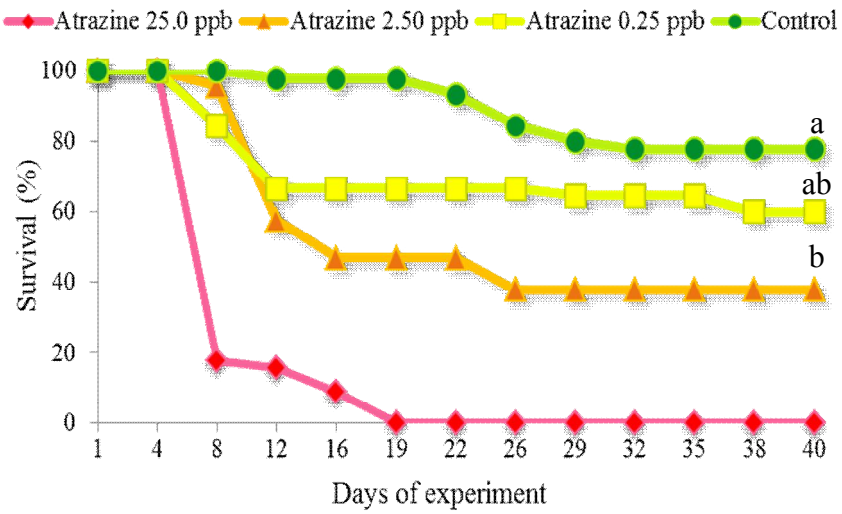

Figure 1. Survival of *H. rugulosus* tadpoles exposed to various concentrations of atrazine for 40 days as compared to control group (a,b represent significant difference at $P < 0.05$.)

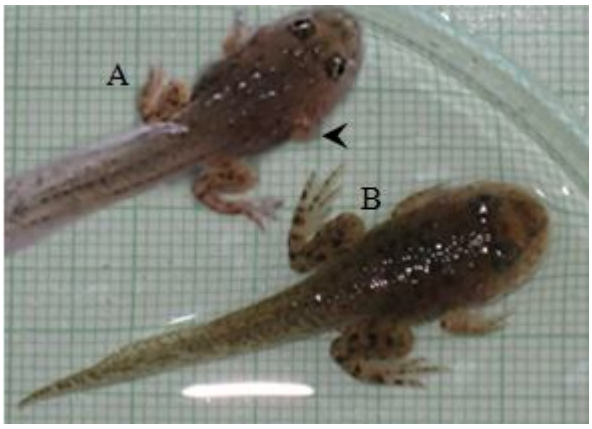

Figure 2. Limb deformity of *H. rugulosus* tadpoles exposed to 2.5 ppb concentration of atrazine for 40 days (A) as compared to control group (B)

factor, the variation of chemical formulation might play an important role in the mortality of amphibians. For example, reports on the toxicity of different atrazine formulations on *Bufo americanus* indicated that the LC_{50} of commercial atrazine (40.8% purity) was 10.7 ppm [17] while the LC_{50} of technical atrazine was as high as 48 ppm [18]. However, an experiment on *Ptychadena bibroni* using commercial atrazine [30] displayed a degree of atrazine sensitivity similar to that obtained in our study of *H. rugulosus*: atrazine at a concentration of 23 ppb could be fatal to *P. bibroni* tadpoles. When atrazine is applied, it is not used as a pure active ingredient but in the form of commercial formulations. The interaction of atrazine and other adjuvants in the commercial formula could be the cause of increased atrazine toxicity and it is this form of atrazine that is commonly used in agricultural fields. Thus, to investigate the direct effect of atrazine on non-target organisms, further experiments are needed on its commercial formulation.

Despite age and chemical formulation factors, species difference in sensitivity to pesticide was also suggested. Hayes *et al.* [19] and Oka *et al.* [31] reported that *X. laevis* could survive high concentrations (up to 200 ppb) of atrazine. Langerveld *et al.* [32] further reported that a significant

Maejo Int. J. Sci. Technol. **2013**, *7*(Special Issue), 60-71

reduction of *X. laevis* survival occurred only at a relatively high concentration (400 ppb) of atrazine exposure. In contrast, a study of *Rana pipien* revealed a marked reduction in survival of tadpoles exposed to only 1.8 ppb of atrazine [33]. The differences between our results and those of others are thus not surprising as a wide range in species sensitivity to pesticides has been described for amphibians [34-35]. *R. pipiens* was also reported to be more sensitive to fungicide (mancozeb) than *Bufo americanus* [36]. The authors also suggested that in a pesticide toxicity test the survival rate of these two species might vary substantially with their developmental stage. The results of atrazine vulnerability obtained from this study suggest that *H. rugulosus*, the domestic frog of Thailand, may be at risk from exposure to the commercial formulation of atrazine even at a relatively modest exposure.

Metamorphosis

The delay of growth and development was observed in tadpoles exposed to atrazine and it was evident in a concentration-dependent manner (Figures 3-4). As shown in Figure 4, at the end of the experimental period of 40 days, none of the larvae exposed to atrazine at the concentration of 2.5 ppb could complete metamorphosis. The emergence of hind limbs (TH) was the most progressive stage of metamorphosis found in the larvae of this group and only few of them could proceed to this stage. Only ten per cent of larvae with complete metamorphosis were seen in the group exposed to a lower concentration of atrazine (0.25 ppb). They also required a longer time than the control larvae to reach the stages of fore limb emergence (TF) and tail resorption (TC). However, the previous study revealed that *H. rugulosus* tadpoles that were exposed to up to 1,000 ppb of pure atrazine did not decrease in size or weight at the end of their metamorphosis [23]. Chemical formulation may be considered to be an important factor for this difference in results. Similar studies showed that *Bufo americanus* treated with 200 ppb of atrazine (40.8% purity) decreased in size and thus the metamorphosis and development rate was indirectly delayed [37], while Freeman *et al.* [38] showed that an exposure of up to 1,000 ppb of atrazine (99.4% purity) did not reduce the frog's size at the end of metamorphosis. While the actual timing of metamorphosis was not recorded in the previous study with *H. rugulosus* [23], a longer period of metamorphosis of atrazine-treated larvae was found in this study.

It is known that anuran metamorphosis is in part regulated by the thyroid hormone. The sequential response of tissues to different concentrations of the thyroid hormone and the development of a negative feedback loop in hypothalamo-hypophyseal-thyroidal axis are needed for a series of changes during metamorphosis [39]. Development of forelimb and resorption of the tail were found to be associated with a surge of the thyroid hormone [40-41]. Several studies show effects of pesticides on anuran metamorphosis and those pesticides are suggested as thyroid disruptor agents. Exposure of *Rana temporaria* tadpoles to 252 µg/l of the fungicide prochloraz caused a delay in metamorphosis and histological changes of the thyroid gland [42]. The decreased expression of thyroid hormone receptor gene (TRbeta) in the tail of *R. temporaria* tadpole exposed to organochlorine pesticide metabolite, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene, was associated with a slowed tail resorption rate [43]. The decreased developmental rate and metamorphosis of *H. rugulosus* larva found in this study might thus be a consequence of atrazine impact on the thyroid axis or the thyroid gland at the cellular and molecular levels.

Maejo Int. J. Sci. Technol. **2013**, *7*(Special Issue), 60-71

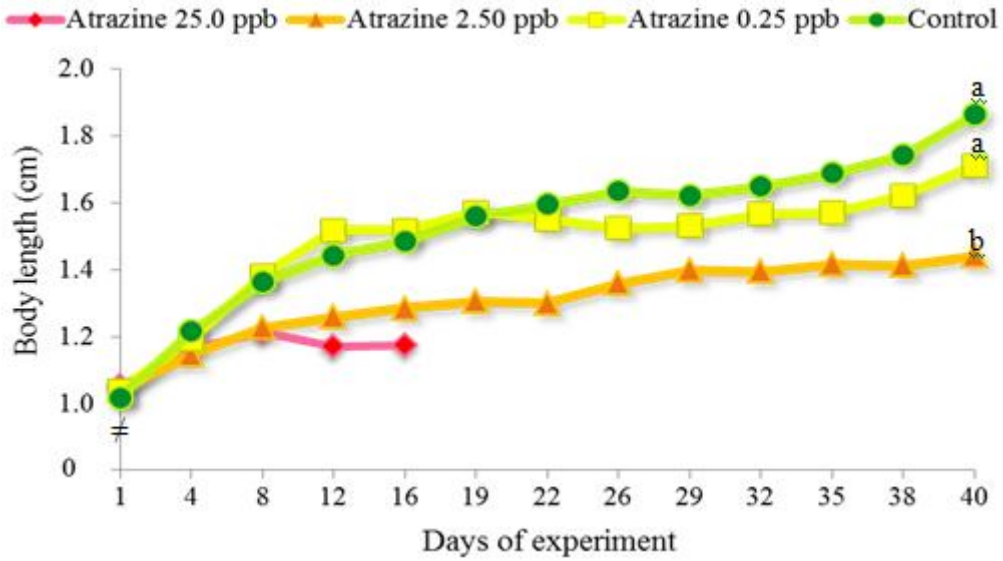

Figure 3. Body length (snout to anus) of *H. rugulosus* tadpoles exposed to various concentrations of atrazine for 40 days as compared to control group (a,b represent significant difference at $P < 0.05$.)

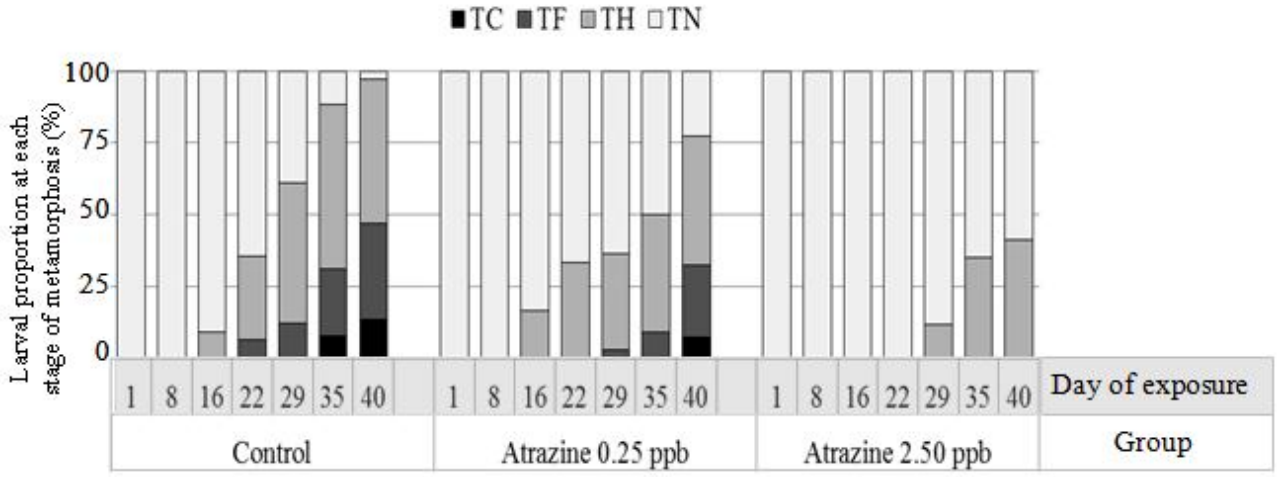

Figure 4. The developmental stages of *H. rugulosus* tadpoles exposed to various concentrations of atrazine for 40 days as compared to control group (TN= tadpole with no limb, TH= tadpole with hind limbs, TF= tadpole with four limbs, TC= tadpole with complete tail resorption)

Gonadal Development and Sex Ratio

The results of gross gonadal morphology and histological examination were used together for sex determination (Figure 5). Morphologically, ovaries of *H. rugulosus* are long, segmented, curl and are covered with melanocytes, and their length reaches over half of the kidney length. The length of testes, however, is limited to only a half of the kidney length or less. The testes are not obviously segmented and do not curl, and their surface is relatively smooth and not covered with melanocytes (Figure 5). It was shown from our results that the larvae of the control group had ovaries or testes with normal gross morphology. The percentage of males in the control group was 36.67%, which was higher than those of morphologically normal males in the atrazine-exposed groups (Figure 6). Exposure to atrazine did not markedly alter the percentage of the females, but it

Maejo Int. J. Sci. Technol. **2013**, *7*(Special Issue), 60-71

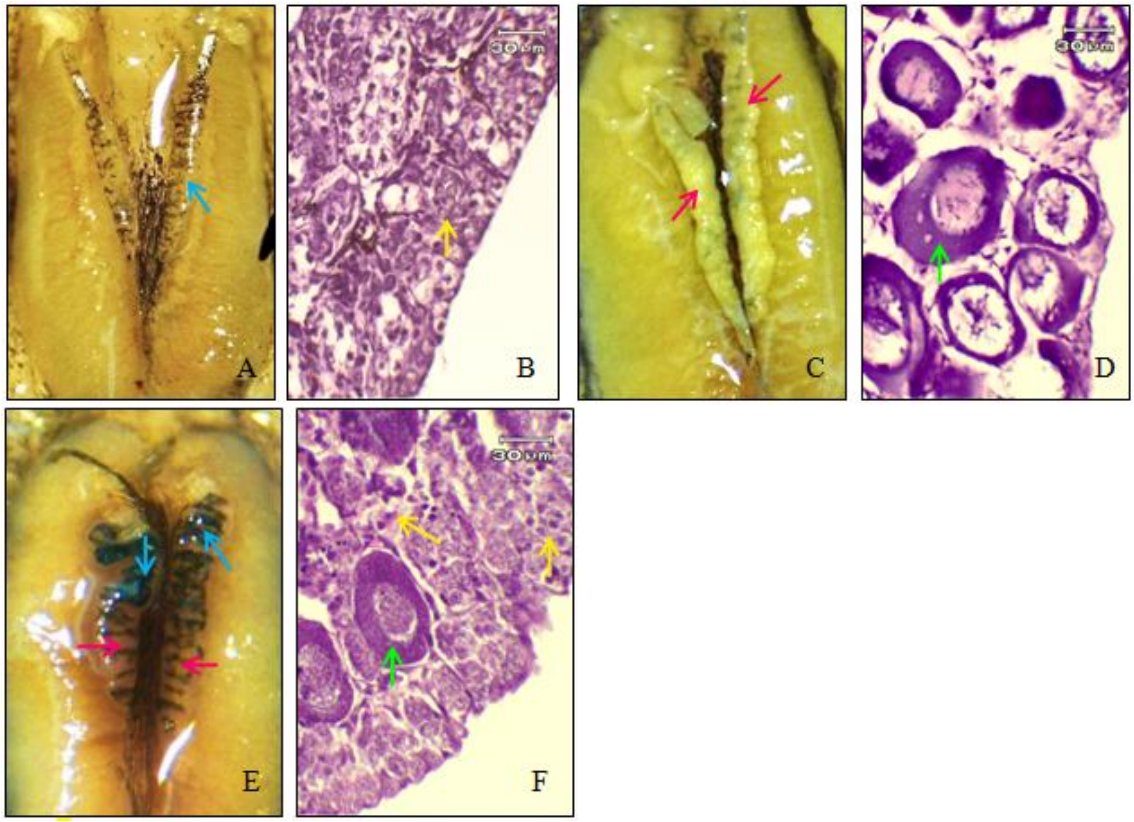

Figure 5. Criteria of gonadal identification and histological examination: (A) gross morphology of male gonad with prominent lobe-shaped and smooth surface testes (↑); (B) H&E stained section of testes with seminiferous tubule (↑); (C) female gonad with long flat-shaped and rough surface ovaries (↑); (D) H&E stained section of ovary with oocyte (↑); (E) Ovotestiscular gonad consisting of both lobe shape and flat shape; (F) H&E stained section of ovotestis showing both seminiferous tubule and oocyte

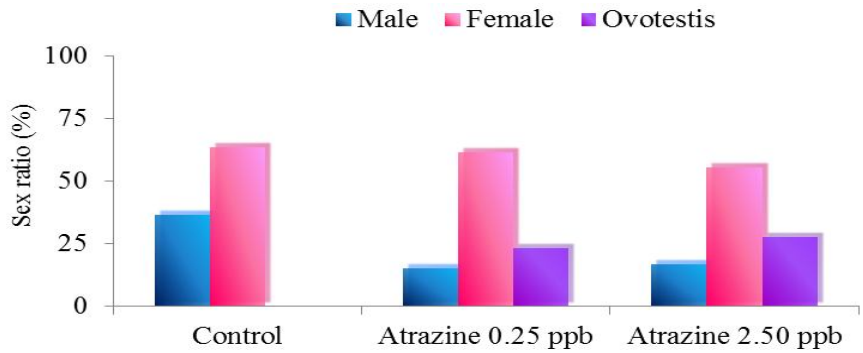

Figure 6. Sex ratio of *H. rugulosus* tadpoles exposed to various concentrations of atrazine for 40 days as compared to control group (a,b represent significant difference at $P < 0.05$.)

Maejo Int. J. Sci. Technol. **2013**, *7*(Special Issue), 60-71

induced feminised testes in the gross morphology as evident by the occurrence of intersex larvae with ovotestes (Figures 5 and 6). It is obvious that the atrazine formulation used in this study induced gonadal feminisation in *H. rugulosus*. Feminisation induced by atrazine was confirmed by histological study. In sections of ovotestes, oocytes were found to be interspersed in the testis tissue which contained mostly seminiferous tubules (Figure 5).

Demasculinised anuran induced by atrazine has been well documented in the literature [19, 21, 44]. Although atrazine itself was reported to exhibit no estrogen activity [45], its activity as a sex hormone disruptor has been proposed [19-21]. The mechanism of action leading to the endocrine disruption by atrazine is likely to be the induction of aromatase, leading to a disturbance in steroidogenesis [19]. Nevertheless, the inability of atrazine to stimulate aromatase gene expression was claimed in *X. laevis* [33]. However, aromatase is a member of the cytochrome P450 (CYP) enzyme family that processes in the steroidal hormone and xenobiotic metabolism [3]. Therefore, the CYP which was reported to be activated by atrazine in mice [46] might interact with the steroidal hormone metabolism pathway and eventually result in an ovotesticular gonad. However, the gonadal abnormality was not observed in the previous study on *H. rugulosus* [23]. The differences between our results and those in the earlier study might be, again, a consequence of the different chemical formulations of atrazine. The pure atrazine might not have had an opportunity to make itself fully apparent in interfering the gonad development, both morphologically and histologically, as did the commercial atrazine. To confirm the effects of pure atrazine on the frog's gonads, additional investigations into the expression of genes and protein markers for each sex are necessary.

CONCLUSIONS

Our study has demonstrates that concentrations of atrazine herbicide used in Thailand can be lethal to *H. rugulosus* larvae. The results indicating a slow growth rate, delayed metamorphosis, demasculinisation and the induction of intersex suggest that *H. rugulosus* larvae inhabiting agricultural areas are a high-risk population. The interaction between the substances in atrazine formulation is proposed as the key mechanism underlying its toxicity. It is hoped that the present investigation will serve as a motivation for the reduction of pesticide use and for an increased attention on amphibian conservation in Thailand.

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