Effects of estrogenic exposure on sex reversal and growth of common lowland frog (*Rana rugulosa*)

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ABSTRACT

During metamorphosis, gonad differentiation may be sensitive to steroid disruption. The common lowland tadpoles (*Rana rugulosa*) were exposed to 17 -estradiol (E2) at the nominal concentrations: 0 (control), 1, 10 and 100 µg/L by immersion, and 0 (control), 1, 10, and 100 mg/feed 1 kg by oral administration method until the end of metamorphosis. The gonad development sex-ratio and growth were determined at complete metamorphosis and 6 months old adults. The highest dose by immersion had a highly significant female biased sex ratio (74 %). Oral administration of E2 did not alter sex ratio. Histological studies showed gonad differentiation (normal ovaries, normal testis, and intersex) at 40 days. The lowest growth found in the 100 µg/ L concentration may result from endocrine function in the reproductive or other systems. At 1 year old adults, disappearance of the oviduct and abnormal oviduct were found in adults in the highest concentration of E2 treatment. Females grew faster than males. When delivered by immersion, high doses of E2 stimulated growth of males.

INTRODUCTION

Culture of the common lowland frog (*Rana rugulosa*) has become an important economic activity in many countries in Southeast Asia. Production in Thailand fluctuated, from 1,010 t in 1999, to 1,944 t in 2004, then 1,535 t in 2007 (Department of Fisheries 2008). Production of this species is increasing and frogs can be grown in a limited space, but profit margins are still low (Somsueb & Boonyaratpalin 2001). Additionally, production is one of the lowest compared with other freshwater species production. The main problem may be the lack of mature females in the spawning season and low survival rate of tadpoles during metamorphosis. In nature, female frogs are bigger than male frogs. Bigger frogs bite the smaller frogs resulting in low survival rate. Thus, the acceleration of female production and survival rate improvement of tadpoles may be potential solutions for this situation.

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Sexual dimorphism varies in frogs. In the case of frog *Adelotus brevis*, males are bigger than females (Katsikaros & Shine 1997). In many anuran amphibians, females have larger body mass but males have much larger stronger forelimbs which is believed to be an adaptation for amplexus (Peters & Aulner 2000).

Gonadal differentiation of amphibians is a steroid-dependent process (Hayes 1997) determined by endogenous sex steroids, and can be altered by treatment with exogenous sex steroid hormones during a critical period of sex differentiation. Many studies have demonstrated that sex steroids can induce the phenotypic sex of tadpoles during metamorphosis, e.g., leopard frog (*Rana pipiens*) (Richards & Nace 1978), bicolored frog (*Rana curtipes*) (Saidapur *et al*. 2001), and wrinkled frog (*Rana rugosa*) (Shibata *et al*. 2002; Iwade *et al*. 2008). Among several types of sex steroids, estrogen can effectively induce the female-biased sex ratio (feminization) of amphibians, resulting in complete sex reversal or gonadal intersex (Bevan *et al*. 2003; Rankouhi *et al*. 2005; Hogan *et al*. 2008).

Estrogen plays a vital role in supporting sexual differentiation and development. Larval development primarily is controlled by the thyroid hormone system, which in turn can be modulated by estrogen (Hayes 1997). Most evidence suggests that estrogens depress thyroid status, thereby inhibiting metamorphosis. Administration of estrogen reduced plasma thyroid hormone concentrations in frog (Vandorpe *et al*. 1989), reduced survival and inhibited growth and development (Hayes 1997; Gray *et al*. 1990; Hayes *et al*. 1993). During the critical developmental period of tadpoles, exposure to exogenous estrogen hormone may disrupt gonadal differentiation. Among all types of estrogen, E2 has shown to be an effective feminization in amphibians. Numerous studies revealed the detectable effects of E2 on female-biased sex ratio depending on several factors such as species (Mackenzie *et al*. 2003), types of hormone (Mackenzie *et al*. 2003; Rankouhi *et al*. 2005), level of concentration (Pettersson *et al*. 2006), and timing of exposure (Richards & Nace 1978; Hogan *et al*. 2008). Additionally, the method of administration, immersion, oral administration, and implantation may also affect on the success of sex-reversal (Hayes *et al*. 1993), due to the absorption of steroid across the skin of tadpoles.

Limited information is available on the reproductive development and control of sex differentiation by exogenous steroid hormone in the common lowland frog. Our objective was to induce sex reversal to phenotypic females with exogenous estrogen by oral administration and immersion during metamorphosis of tadpoles. Sex reversal and sex differentiation were detected by histological studies during metamorphosis, while the characteristics of sexual morphology and sex ratio were monitored at 6 month old frogs. The survival rate was counted at the end of metamorphosis and 6 month old individuals. Overall, results from this study should address understanding of how sex differentiation responds to the different concentrations and methods of exogenous estrogen and how the alterations of sex differentiation affect survival rate of tadpoles and mature frogs.

MATERIALS AND METHODS

Samples

Rana rugulosa larvae were bred at Tak Inland Fisheries Research and Development Center Hatchery. Mating was induced by injecting 10 µg of gonadotropin-releasing hormone analogue (Sigma) mixed with 10 mg of domperidone (Janssen-Cilag) into the hind limb of common lowland frog broodstock. The naturally fertilized eggs were obtained from 12 pairs of frogs.

Experiment 1

To investigate responses of sex differentiation to different concentration levels and administration methods of E2 (Sigma), the long-term E2 exposure to larvae was initiated within 2 days after hatching. The experiment was divided into triplicates of 2 treatments. The 300 tadpoles were randomly transferred to round cement tanks (1 m diameter) which contained 100 litres of water per replicate. The first four treatment groups were immersed the whole time at the four nominal concentrations: 0, 1, 10 and 100 µg E2/l. Another four treatment groups were fed with the pellet feed mixed with E2 at 0, 1, 10, and 100 mg/feed 1 kg. All treatments were fed with powder feed at the age of 2-5 days old, after which they were fed with floating pellet feed (about 1-2 mm) with protein of not less than 37 %, by apparent satiation thrice daily for 38 days. Concentrated stock solutions of E2 for oral administration were prepared in ethanol and sprayed to mix the feed to achieve the nominal concentration: 0, 1, 10, and 100 mg/feed 1 kg. The ratio of ethanol per feed was 0.25 l/feed 1kg. Each treatment consisted of three replicate tanks in a static-renewal system. Water quality was monitored weekly. The ranges of water quality parameters were as follows: temperature at 26.2-27.1°C, pH of 7.2-7.9, dissolved oxygen at 7.7-8.0 mg/L, 115.2-136.0 mg $CaCO_3$/L alkalinity, 68.5-79.7 mg $CaCO_3$/L hardness, and 0.04-0.11 mg NH_3-N/L ammonia. Concentrated stock solutions of E2 for immersion were prepared in ethanol and diluted in water to achieve the nominal concentrations: 0, 1, 10 and 100 µg E2/l. The uneaten food and waste were removed when the water was changed 100% daily. The exposure period of all treatments were terminated at the end of metamorphosis (~40 days). After E2 exposure, frogs were allowed to develop further in round cement tanks (1 meter diameter) without E2 until 6 months old.

On termination of the experiment, the juvenile frogs were anesthetized by immersion in 0.1% MS-222 solution for measuring weight and length. The survival and abnormality rates of tadpoles were recorded. The remaining tadpoles continued developing to the adult stage.

Experiment 2

To monitor the gonad development, growth, and survival rate in the adult stage, 150 tadpoles in each treatment were assigned to a new tank for 6 months (~ 155 days) in untreated-medium. The juvenile individuals were fed ad libitum daily with pelleted food. Water quality was monitored weekly. Water quality parameters were as follows: temperature at 26.7-27.3°C, 7.3-8.0 pH, 3.4-8.0 mg-O_2/L, 127.5-163.4 mg

$CaCO_3$/L alkalinity, 76.2-105.8 mg $CaCO_3$ /L hardness, and 0.04-0.18 mg NH_3-N/L ammonia. The uneaten food and waste were removed when the water was renewed 100 % daily. At the end of experiment, the adult individuals were euthanized in 0.1% MS-222 and sacrificed to examine sexual morphology and sex ratio visually. Gonadal maturity was determined by gonadosomatic index (GSI). Additionally, the body weight, standard length, and survival rate of adult frogs were recorded.

Gonadal differentiation and histological studies

During the exposure period (experiment 1), six tadpoles per treatment were collected every 4 days after hatching (at the ages of 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 days) 10 times and fixed in 70 % ethanol until sectioning. The stages of sex development and differentiation (male, female, and intersex) were observed by histological evaluation of gonads. The tissue samples were dehydrated in a series of graded ethanols and embedded in paraffin. The sections of tissues were stained with haematoxylin-eosin and mounted with Permount (Fisher Scientific). The stained sections were examined under a light microscope (Nikon).

Data analysis

The body weight and length of juvenile and adult frogs following immersion and feeding exposures were analyzed statistically using analysis of variance (ANOVA) followed by the Tukey-Kramer's multiple comparisons test. Normality of data was tested by using Shapiro-Wilk normality test and homogeneity of variance was monitored by Bartlett's test. Survival rates were arcsin transformed before analysis and analyzed to compare the treatment and control groups. The percentage of sex ratio was analyzed with chi-square goodness-of-fit test to deviations from assumed ratio of 1:1 (Goleman *et al*. 2002). Phenotypic females, males, and intersex were determined for analysis based on gonad morphology. Due to the low number of observations, the Fisher's Exact test was used to determine differences of sex ratio between treated and control groups. Statistical significance of all tests was set at ≤ 0.05.

RESULTS

Sex ratio and gonadosomatic index

The percentage of female frogs from immersion were 40, 46, 42 and 74 %, respectively (immersed at 0, 1, 10 and 100 µg E2/l respectively) and oral administration were 38, 40, 38 and 38 % respectively (feed mixed with E2 at 0, 1, 10, and 100 mg/feed 1 kg respectively). At the highest dose of immersion (100 µg/l), the percentage of females was higher (74 %) when compared with the other treatments and the control ($p \leq 0.05$, ANOVA), while the apparent intersex was detected in the 1 and 10 µg/l of immersion and 1 mg/feed 1 kg of oral administration. In the case of oral administration, there were no differences in sex ratio among the treatments and the control. The gonadosomatic index (GSI) of male tadpoles immersed in E2 at 10 and 100 µg/l were higher ($p<0.001$) than that of controls (Table 1).

Table 1 Effects of estrogen exposure by different methods on sex ratio (percent) and % GSI (mean±SD) in common lowland frog (*Rana rugulosa*) at 155 days.

Treatment	Sex ratio			% GSI		
	Female[1]	Male[1]	Intersex[1]	Female[2]	Male[2]	Intersex[3]
Immersion						
0 µg/l (control)	40[a]	60[b]	0	0.165±0.037[a]	0.005±0.003[a]	-
1 µg/l	48[a]	50[b]	2	0.182±0.068[a]	0.017±0.014[ab]	0.0066
10 µg/l	42[a]	56[b]	2	0.191±0.067[a]	0.024±0.005[b]	0.0045
100 µg/l	74[b]	26[a]	0	0.210±0.113[a]	0.026±0.008[b]	-
Oral administration						
0 mg/feed 1 kg (control)	38[a]	62[b]	0	0.192±0.048[a]	0.020±0.010[b]	-
1 mg/feed 1 kg	40[a]	56[b]	4	0.174±0.069[a]	0.022±0.007[b]	0.019±0.001
10 mg/feed 1 kg	38[a]	62[b]	0	0.172±0.074[a]	0.019±0.007[b]	-
100 mg/feed 1 kg	38[a]	62[b]	0	0.205±0.051[a]	0.020±0.009[b]	-

[1] Results are in percentage. Treatments were analyzed with chi-square goodness-of-fit test to determine deviations from assumed ratio of 1:1 and different superscript letter within column were significantly different ($P < 0.05$).
[2] Results show mean±SD. Mean values are significantly different ($P<0.05$) within column from the control with one-way ANOVA and values assigned a different superscript differ with Tukey's multiple range test.
[3] Values are means.

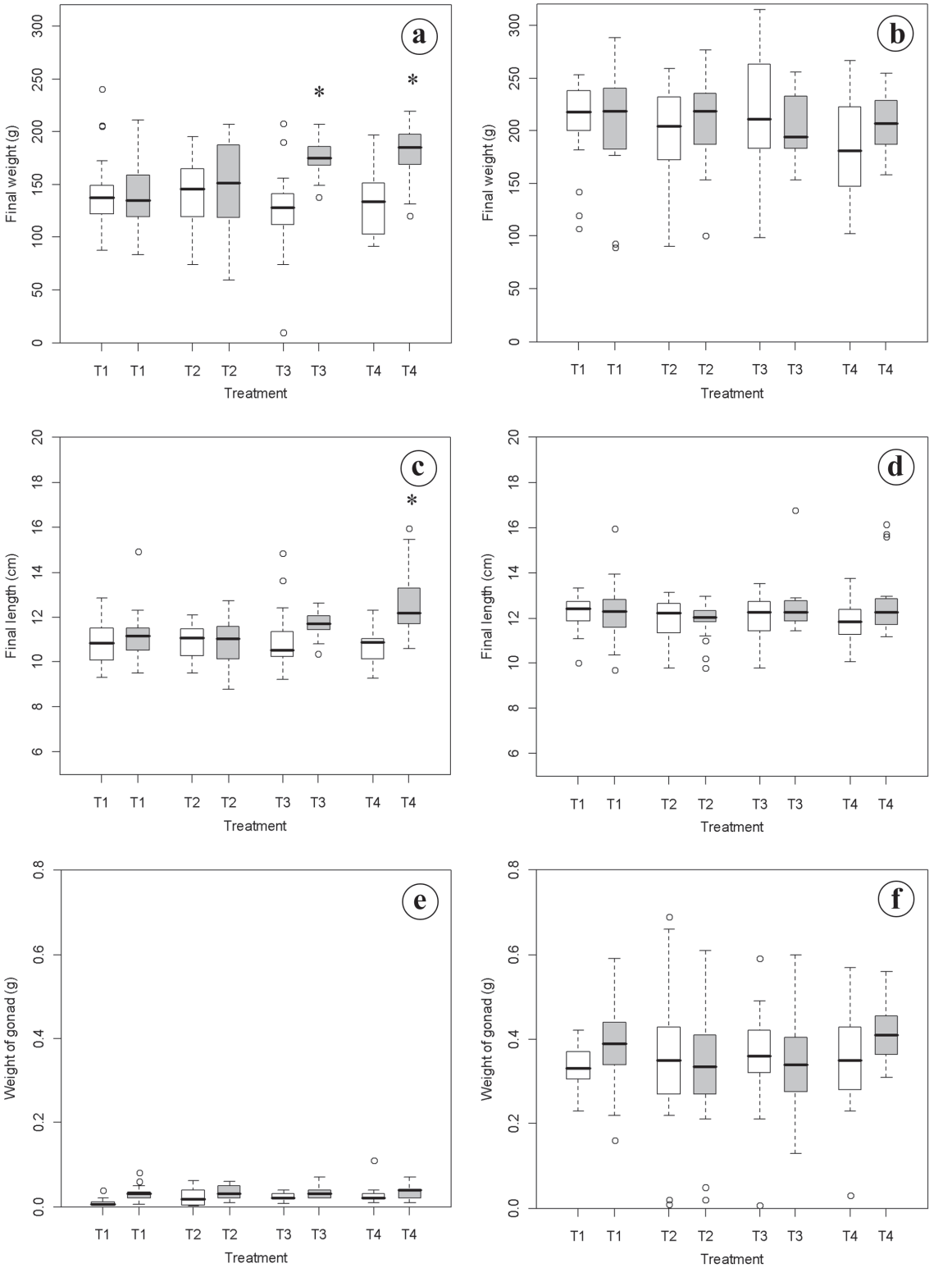
Growth and survival rate

Females frogs grew faster than male frogs in the control and in most treatments (Figure 2). The adult males had a highly significant difference in body weight and length treated with oral administration ($p<0.001$) (Figure 1). There was no effect of any method of estrogen delivery on survival rate ($p>0.05$) (Table 2). There were no treatment-related effects at the end of metamorphosis ($p>0.05$; ANOVA) (Table 2). The body weight of E2-exposed tadpoles with the oral administration and immersion method was not different among treatments and controls. For adult frogs, the body weight of frog immersed in E2 at 100 µg/l showed significantly through the level of estrogen concentration in the immersion when compared with the untreated group ($p<0.001$; ANOVA) (Table 2). There were no treatment-related effects on survival rate at the adult stage ($p>0.05$; ANOVA) (Table 2). When the body weight, body length, and weight and length of gonad were analyzed separately, the growth and gonad development among method of administrations and treatments were significant in male, while there was no estrogen-effect in female (Figure 1).

Table 2 Effects of estrogen exposure on growth (mean±SD) and survival rate (mean±SD) in common lowland frog (*R. rugulosa*) at 40 and 155 days.

Treatment	Experiment 1 (40 days)			Experiment 2 (155 days)		
	Final weight (g)[1]	Final length (cm)[1]	Survival rate (%)[1]	Final weight (g)[1]	Final length (cm)[1]	Survival rate (%)[1]
Immersion						
0 µg/l (control)	3.15±0.21^{a}	3.17±0.07^{a}	54.52±3.20^{a}	157.20±10.48^{b}	10.16±0.27^{ab}	91.33±3.06^{a}
1 µg/l	3.13±0.21^{a}	3.12±0.07^{a}	56.79±2.83^{a}	154.57±8.73^{ab}	10.17±0.18^{ab}	90.67±1.15^{a}
10 µg/l	3.17±0.18^{a}	3.15±0.08^{a}	54.40±4.11^{a}	151.24±6.16^{ab}	10.20±0.16^{b}	90.67±2.31^{a}
100 µg/l	2.93±0.04^{a}	3.06±0.03^{a}	57.14±2.92^{a}	140.52±2.06^{a}	9.83±0.03^{a}	92.67±4.16^{a}
Oral administration						
0 mg/feed 1 kg (control)	3.19±0.47^{a}	3.06±0.13^{a}	58.10±1.15^{a}	151.56±10.99^{ab}	10.03±0.27^{a}	92.67±4.16^{a}
1 mg/feed 1 kg	3.41±0.18^{a}	3.14±0.06^{a}	55.36±3.41^{a}	151.44±6.79^{ab}	9.91±0.24^{a}	92.67±3.06^{a}
10 mg/feed 1 kg	3.38±0.25^{a}	3.11±0.09^{a}	58.93±2.58^{a}	151.96±6.13^{ab}	9.94±0.12^{a}	92.00±4.00^{a}
100 mg/feed 1 kg	3.11±0.43^{a}	2.97±0.12^{a}	58.69±4.41^{a}	151.11±7.01^{ab}	9.93±0.15^{a}	91.33±4.16^{a}

[1] Results are mean±SD. Different superscripts show significant difference as determined by one-way ANOVA (P<0.05) followed by Tukey's multiple range test.


a
b
c
d
e
f
Final weight (g)
Final length (cm)
Weight of gonad (g)
Treatment
T1 T1 T2 T2 T3 T3 T4 T4
0 50 100 150 200 250 300
6 8 10 12 14 16 18 20
0.0 0.2 0.4 0.6 0.8
*

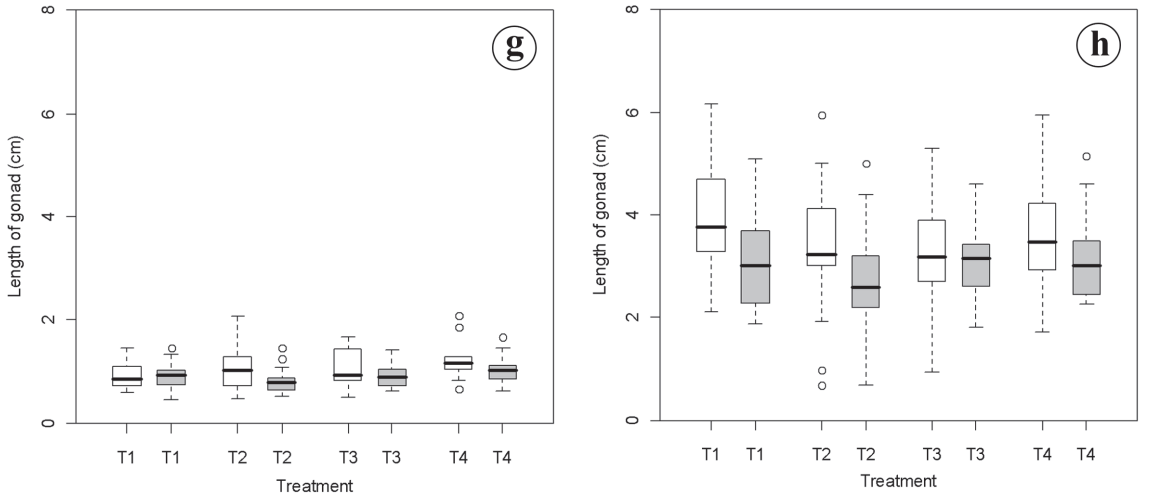


Figure 1. The growth and gonad parameters of frog the age of 155 days.
The male frogs show in (a,c,e and g) and the female frogs show in (b,d,f and h).
immersion method (white) : T1 (control (no estrogen)), T2 (1 µg/l)
T3 (10 µg/l) and T4 (100 µg/l)
oral administration method (grey) : T5 (control (no estrogen)), T6 (1 mg/feed 1 kg.)
T7 (10 mg/feed 1 kg.) and T8 (100 mg/feed 1 kg.)
Significant effects due to the estrogen exposure were determined by one-way ANOVA and Tukey's multiple range test (*; $P<0.05$).

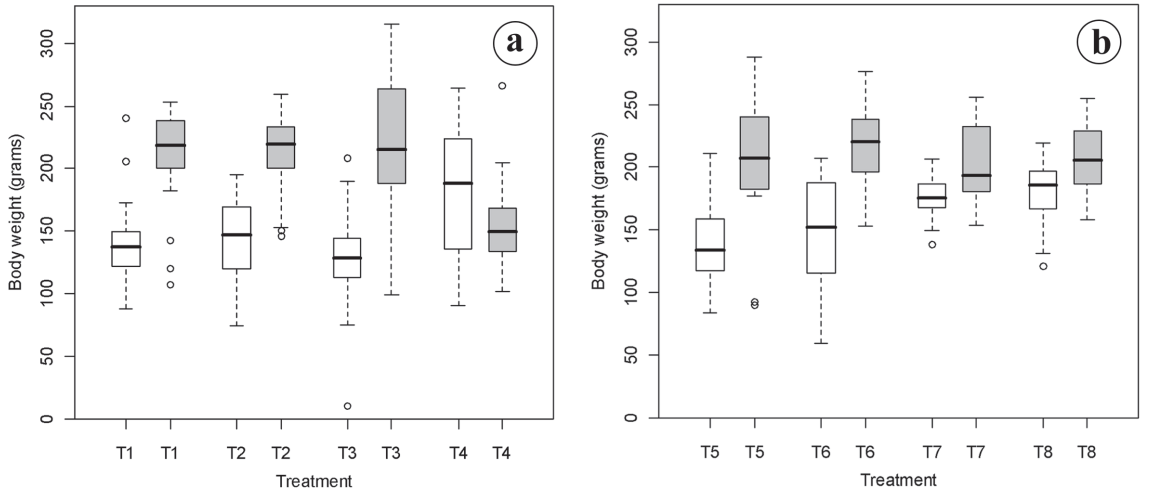


Figure 2. The body weight of common lowland frog at the age of 6 months.
The male frogs show in white graph and the female frogs show in grey graph.
immersion method (a) : T1 (control (no estrogen)), T2 (1 µg/l)
T3 (10 µg/l) and T4 (100 µg/l)
oral administration method (b) : T5 (control (no estrogen)), T6 (1 mg/feed 1 kg.)
T7 (10 mg/feed 1 kg.) and T8 (100 mg/feed 1 kg.)
Significant effects due to the estrogen exposure were determined by one-wayANOVA and Tukey's multiple range test (*; $P<0.05$).

Sex effects

Female frogs grew faster than male frogs (p<0.001) (Figure 2). The high dose of oral administration increased size of male frogs higher than female frogs (p<0.05). The highest rate of immersion reducedfemale weight (p<0.05) (Figure 2). Observed means for GSI in both male and female frogs increased with increasing E2 when delivered by immersion however, the differences were not significantly different (p>0.05) (Table 1).

Histological studies

On the 8th day after hatching, the primordial germ cells were detected in the gonad of tadpoles (Figure 3). An undifferentiated gonad was established. At 40 days, the tadpoles in treated group showed normal ovaries, testis, and intersex (Figure 4) while the untreated group had no intersex. At 1 year old adults, disappearance of the oviduct and abnormal oviduct were found in adults in the highest concentration of E2 treatment.

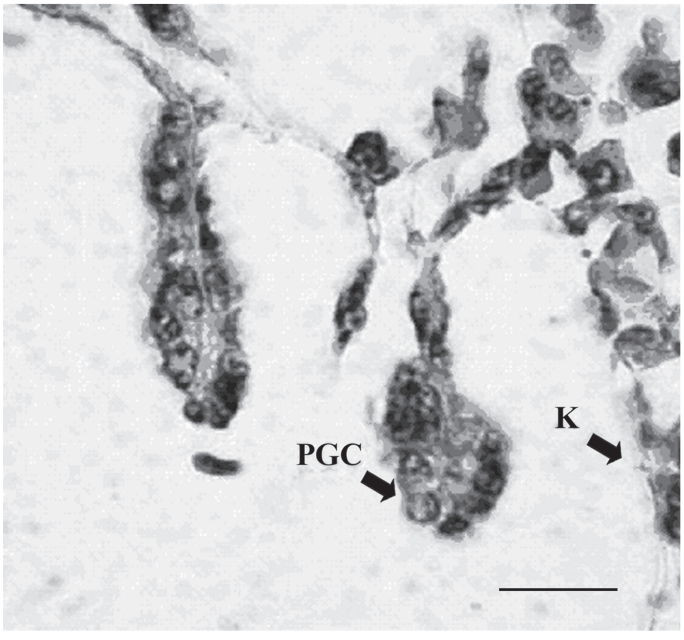


Figure 3. Photomicrographs of gonad of 8 day old *Rana rugulosa*. Gross morphology of gonad from a control frog found primordial germ cell (PGC) and kidney (K) (Bouin's H&E; 1,000) (Bar = 100 μm).

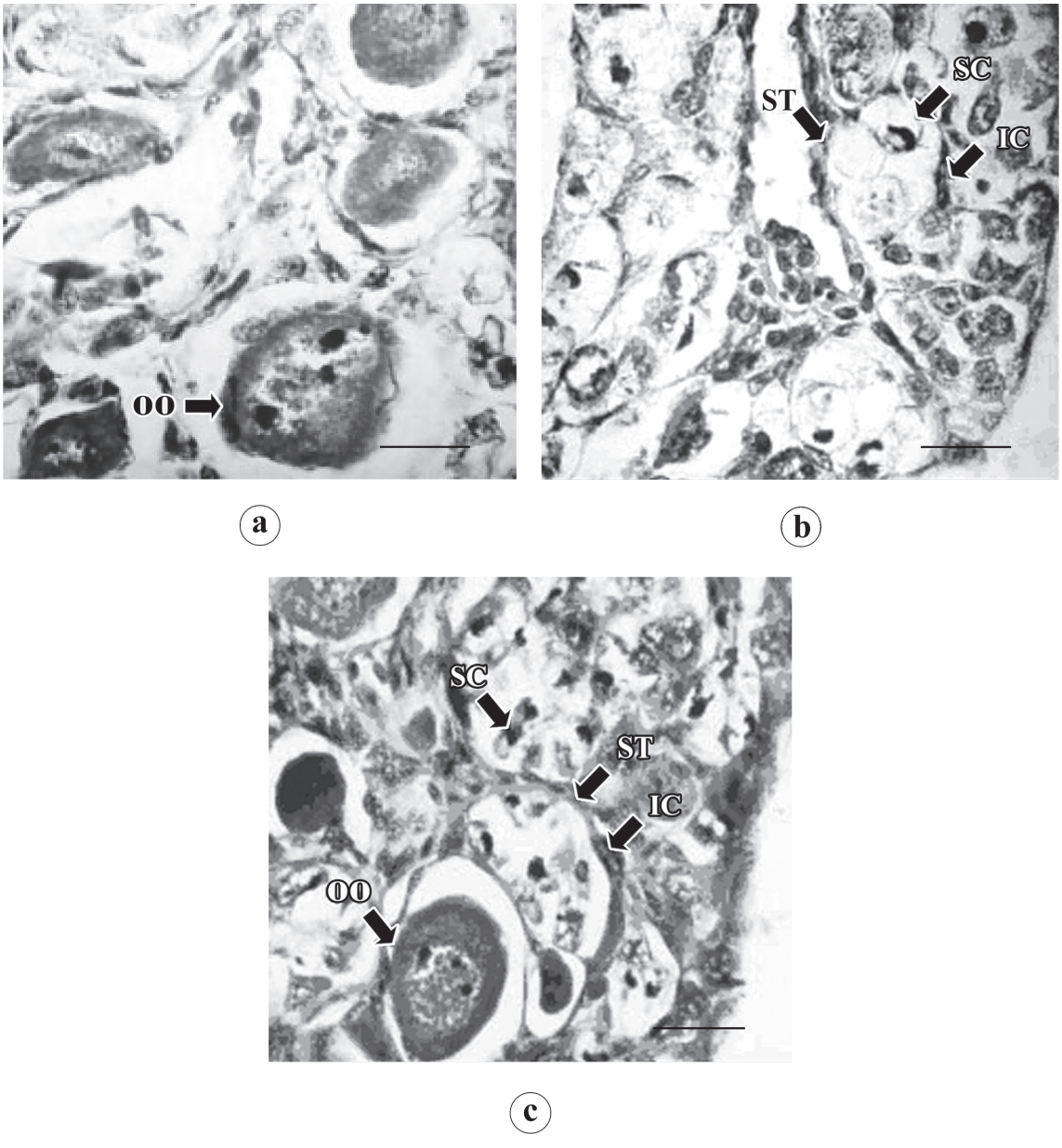


Figure 4. Photomicrographs of gonad of 40 day old *R. rugulosa*.

(a) Gross morphology of ovaries of a control female frog found oocyte (oo) (Bouin's H & E; 1,000).

(b) Gross morphology of testis of a control male frog found seminiferous tubules (ST), interstitial cells (IC) and spermatocytes (SC) (Bouin's H & E; 1,000).

(c) Gross morphology of gonad of a intersex frog exposed to 1 µg/l E2 found oocyte (oo), seminiferous tubules (ST), interstitial cells (IC) and spermatocytes (SC) (Bouin's H & E; 1,000) (a-c, Bars = 100 µm).

DISCUSSION

Development and differentiation of the reproductive system are hormone-dependent processes. Exogenous estrogen caused reproductive alterations and sex-reversal during larval development as indicated by histological and phenotypic changes. Only the highest concentration (100 µg/l) administered as a bath can stimulate feminization (74 %). The dose of estrogen required for feminization in the common lowland frog was higher than that for the larval clawed frog (*Xenopus tropicalis*) and *Rana pipiens* for which exposure to 0.3-30 and 1-10 µg/L estrogen resulted in female biased sex-ratios and intersex conditions at metamorphosis (Mackenzie *et al*. 2003; Pettersson *et al*. 2006). Additionally, the temporary sex reversal may have occurred in the common lowland tadpoles at low doses resulting in no permanent feminization. Chang (1995) observed and explained this phenomenon in estradiol-treated larvae of *Bufo americanus* which can be changed to female during metamorphosis and subsequently reverted back to male and intersex.

The variation of individual responses to E2 may depend on species and method of administration. Several studies showed that the different response of genus *Rana* to estrogen, e.g., the estrogen can stimulate feminization in *R. pipiens, R. catesbiana* and *R. esculenta,* but there was no effect of estrogen on sex-reversal in *R. nigromaculata* and *R. clamitans* (Foote & Witschi 1939; Puckett 1939; Iwasawa & Kobayashi 1974; Rastogi & Chieffi 1975; Mackenzie *et al*. 2003). In the previous studies, there were various methods of exposure to the steroid hormone, e.g., subcutaneous injection, oral administration, and immersion, which may have caused variation in the results from one species to another.

In this study, the group treated by dissolving estrogen in the water stimulated higher feminization than mixing hormone in the feed. This phenomenon may be explained by the fact that frogs usually take water only through ventral skin, and male frogs absorb more water than female ones (Hayes *et al*. 1993, 1995 ; Iguchi *et al*. 2001). Therefore, the hormone can easily pass into the circulation system in the former method.

Many factors such as environment and age may affect the efficacy of estrogen on the feminization, e.g. the temperature can inhibit the function of estrogen which controls growth and development in *Bufo boreas* (Hayes *et al*. 1993) and the stage 52 of larval development is the latest stage for which feminization can still be accomplished (Hayes 1997). Additionally, the variation of estrogen responses depends on exposure period. This phenomenon is explained by Villapando & Merchant-Larios (1990) as they observed that the sex-reversal of clawed toad (*Xenopus laevis*) appeared complete when the sex steroid was administered before translocation of primordial germ cells from the gonadal epithelium into the medullary region. In the northern leopard frog (*Rana pipiens*), the tadpoles exposed early (stage 27-30) in development had a strong female-biased sex ratio when compared to the controls (Hogan *et al*. 2008).

At one year old, the oviduct and abnormal oviduct disappeared in adults at

the highest concentration of E2 treatment. This is in agreement with a study on *Xinopus tropicalis*, which lacked oviducts when exposed to high concentrations of estrogen (Pettersson *et al*. 2006), while *Rana temporaria* displayed hypertrophied oviducts after exposure to estradiol benzoate during the larval stage (Gallien 1955). Wallace *et al*. (1999) reported that a common side effect of steroid hormone is a suppression of oviduct growth. Although the estrogen-exposed frogs have normal ovaries, the individuals lacking oviducts will result in sterility.

During metamorphosis, there are many kinds of hormones which regulate growth and larval development, for example: the level of thyroid hormone can increase throughout larval development (Leloup & Buscagali 1977), sex steroid, especially estrogen can control thyroid hormone activity which simulates growth through hypothalamic and pituitary control (Hayes 1997), and corticoids (corticosterone) that can accelerate tail reabsorption and limb development (Hayes 1995b). The sex steroid was correlated negatively to metamorphic development, e.g., the larval development was reduced in *Rana pipiens* (Richard & Nace 1978) and the tail reabsorption was inhibited in *Xenopus laevis* (Gray & Janssens 1990). Additionally, crowding the tadpoles can inhibit the growth and development and accelerate the levels of corticosterone that resulted in reduced metamorphosis (Hayes 1997).

Female frogs grew faster than males. Hand selection of frogs to raise monosex populations might be beneficial. However, that may not be practical of economical so sex reversal or production of genetically female populations may allow aquaculturist to take advantage of this sexual dimorphism

The present study demonstrated that immersion to 100 µg/l of E2 can induce sex reversal in the common lowland frog. However, only 50% of the males were sex reversed. This treatment increased the growth of males, but unfortunately decreased the growth of females. Thus, the mean body weight of the population decreased counteracting the benefits of feminization. Oral administration of E2 increased the growth of males, but not enough to alter the population mean. E2 given as a bath stimulated gonadal growth. However, oral administration of E2 did not affect gonad size.

Additional dose rates will need to be evaluated to find a treatment that will more effectively feminize the frogs, but the current results indicate that the growth of lowland frogs may be suppressed. Other feminizing agents may be more effective for lowland frogs regarding sex reversal and growth, and should be evaluated. Alternatively, sex reversal and breeding might be evaluated as natural production of monosex female populations might result in the beneficial growth of females without the counteracting growth suppression from the hormone.

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