

Testis-Ova in Male Rice Frog (*Fejervarya limnocharis*) - A Natural Process of Sex Differentiation or a Suitable Biomarker of Effect for Ecotoxicology?

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ABSTRACT. — A previous study found that adult male rice frogs (*Fejervarya limnocharis*) inhabiting agricultural areas with varying levels of herbicide use exhibited testis-ova (TO). However, it remained unclear whether the occurrence of TO is associated with underlying herbicide contamination in agricultural areas or represents a natural aspect of testicular development in this species. This study aimed to examine the gonadal histology and intersex condition of the male rice frog, *F. limnocharis*, living in an agricultural area in central Thailand with no history of herbicide utilization over the last 10 years. The results showed that the incidences of testicular ovarian follicles (TOFs) in adult, subadult, and juvenile frogs were 46/50 (92%), 7/7 (100%), and 6/6 (100%), respectively. Crucially, these TOFs were observed in rice frogs even when herbicide analysis showed paraquat tissue residue below detection limits. These findings suggest that the presence of oocytes within the testis in *F. limnocharis* may represent a natural, transition phase of testicular development in the undifferentiated and semi-differentiated types of the normal sex differentiation process in amphibians. This prompted an evaluation of whether the TOFs could serve as a biomarker for assessing the effect of and susceptibility to herbicide contamination in this frog species.

KEYWORDS: amphibian, endocrine disrupting chemicals, histology, intersex, rice field

INTRODUCTION

Amphibians are widely recognized as bioindicators of environmental health due to their unique ecological and physiological characteristics (Saber et al., 2017). Their reliance on aquatic and semi-aquatic habitats for survival, coupled with their permeable skin that facilitates gas exchange, makes them particularly sensitive to environmental changes and pollutants (Felsemburgh et al., 2007). This sensitivity allows amphibians to detect and respond to subtle environmental anomalies more effectively than many other organisms, making them valuable subjects for monitoring ecosystem integrity and identifying ecological disturbances. Currently, amphibian populations are mostly declining, with one contributing factor being the use of agricultural chemicals, particularly herbicides (Beebee and Griffiths, 2005).

In Thailand, many herbicides are used, such as atrazine, glyphosate, and paraquat. When these substances contaminate the environment, they may cause adverse effects on non-target organisms. This is especially the case for the herbicide atrazine that can disrupt the action of the endocrine glands, such as adjusting the rate of activity of the aromatase enzyme that changes testosterone into the female hormone

estrogen, which leads to those animals developing an intersex condition (Hayes et al., 2002).

Previous reports indicated that herbicides like atrazine, glyphosate, and paraquat were found as contaminants in tissues of the rice frog *Fejervarya limnocharis* (Jantawongsri et al., 2015), while atrazine residue was present in the residing paddy field at Nan Province (Maneein et al., 2011). Since atrazine is known to be an endocrine-disrupting chemical that can affect gonadal development (Hayes et al., 2002), its contamination may lead to sex reversal or intersex gonads of organisms living in the contaminated areas. A prior study revealed that adult male *F. limnocharis* living in agricultural areas with different degrees of herbicide utilization showed testicular ovarian follicles (TOFs) in the testis (Kitana et al., 2018). However, it is unclear whether the presence of TOFs is linked to a background contamination of herbicide in the agricultural areas or the normal developmental process of the testis in this species.

There are three patterns of normal gonadal sex differentiation in amphibian species: differentiated, semi-differentiated, and undifferentiated types (Witschi, 1929). The intersex gonads or testis-ova (TO) can be found during the development of the semi-differentiated and undifferentiated types. The semi-differentiated type has been reported in *Lithobates*

sylvaticus (Witschi 1929), *Rhacophorus arboreus* (Tanimura and Iwasawa 1989), and *Rana curtipes* (Gramapurohit et al., 2000), while the undifferentiated type has been reported in *Bufo bufo* and *R. ornativentris* (Gramapurohit et al., 2000) and *Hoplobatrachus rugulosus* (Trajitt et al., 2020). The gonadal sex differentiation pattern in Thai *H. rugulosus* reared in the laboratory under a non-contaminated condition was of an undifferentiated type, where intersex gonads were found during testicular development. Moreover, oocytes were found in the testis of some adult male frogs alongside spermatogenesis (Trajitt et al., 2020). This inferred that TOs could be found in normal development in some species depending on the sex differentiation pattern. Thus, this study aimed to examine the histological structure of testis in male rice frogs living in an area with no background contamination of herbicide.

MATERIALS AND METHODS

Study site

The Chulalongkorn University Forest and Research Station (CUFRS), located in Kaeng Khoi District, Saraburi Province, central Thailand, covers a total area of 3,362 rai (1 rai = 1,600 square meters). The area is divided into two parts: Part 1 is comprised of 2,630 rai of degraded forest, which the Royal Forestry Department has granted a permit to Chulalongkorn University for academic purposes. Part 2 consists of 732 rai allocated for use by the Chulalongkorn University Alumni Foundation; this section includes one reservoir and two ponds. The CUFRS has remained free of agricultural chemicals for more than 10 years.

Frog collection

During October to November 2018, 63 male frogs were field collected during visual encounter surveys at the CUFRS, Saraburi province, central Thailand. Frogs were euthanized by immersion in 0.5% MS-222 solution (Torreilles et al., 2009). The experimental protocol was approved by the Animal Care and Use Committee of the Faculty of Science, Chulalongkorn University (Protocol Review No. 1923001). The stage of each male frog was classified by their secondary sex characteristic (a black stripe under the chin) into the three groups of adults, subadults, and juveniles (Fig. 1). Frogs with a clear M-shaped black stripe were deemed to be adults since it indicates the presence of a fully developed vocal sac. In subadult male frogs, the dark stripes are not yet clearly M-shaped. The black stripes originate from the sides of the mouth and then meet in

the middle. Juvenile frogs could not be classified by secondary characteristics. However, confirmation of sex requires study of the primary characteristics following dissection and investigation of the character of the gonads.

Data collections

The snout to vent length (SVL) and body weight (BW) of each frog were recorded. After euthanization, the frogs were dissected and the weight of testes of each individual was measured. The organs were preserved in Davidson's fixative.

Assessment on herbicide contamination

To determine background contamination of herbicides in frog tissue, frog samples were collected in November 2018 and kept immediately at 4°C. The frog samples were freeze-dried (Epsilon 2-4 LSCplus) until complete dryness and measured for dry weight (Ohaus Pioneer Analytical Balances; accuracy 0.0001 g). Six samples ($n = 1$ for juvenile; $n = 1$ for sub-adult, $n = 4$ for adult) were extracted for herbicide residues. In this study, paraquat, the most popular herbicide used in central Thailand, was selected as a representative of herbicide for analysis. The extraction of paraquat follows the modified protocol of Brown et al. (1996) and Quick, Dyson, and Holliman (1990). Briefly, 100 mg of lyophilized tissues were priorly mixed with 200 μ L of ultrapure H₂O, added with 100 μ L of hexane (Merck) and 600 μ L of 10% trichloroacetic acid (TCA), and shaken thoroughly. After centrifugation at 2,000 xg for 15 min, 500 μ L of aqueous phase was transferred into clean tube as a first extract. The remaining samples were added again with 250 μ L of 10% TCA and shaken thoroughly. After centrifugation at 2,000 xg for 15 min, 200 μ L of aqueous phase was transferred into the first extract, thereby, a total extract of 700 μ L of aqueous phase was obtained. The extracts were added with 400 μ L of hexane and shaken thoroughly. After centrifugation at 2,000 xg for 15 min, 600 μ L of aqueous phase was collected. To adjust the pH to 7.0, extracts were added with 100 μ L of 2 M Tris-basic buffer and shaken thoroughly. After centrifugation at 2,000 xg for 5 min, a total extract of 500 μ L of aqueous phase was transferred into clean tube and labelled as an aqueous extract. The remaining precipitated samples were added with 200 μ L of ultrapure H₂O as precipitated extract. To evaluate for recovery of extraction, 1.5 ng of standard paraquat (200 μ L of 7.5 ng/mL paraquat solution; Abnova, Taoyuan City, Taiwan) was added to a representative sample (as a spiked sample).

Enzyme-linked immunosorbent assay (ELISA) kit for paraquat determination was obtained from Abnova (Taoyuan City, Taiwan), and the methods were after the manufacturer's protocol. Briefly, duplicated

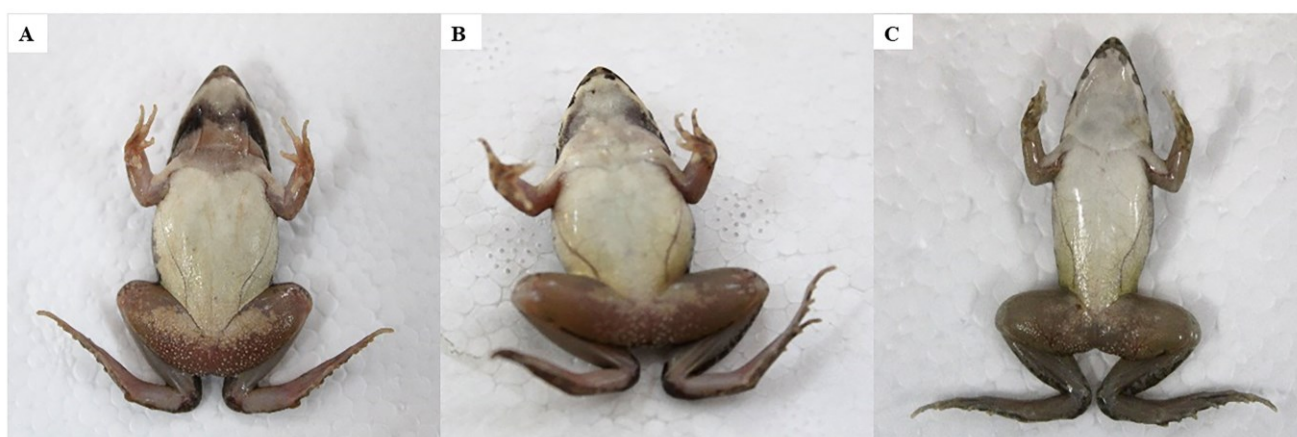


FIGURE 1. Classification of male frogs at different stages based on the colored stripes under their chins as (A) adult, (B) subadult, and (C) juvenile.

addition of 25 μ L of samples, spiked sample, and standard paraquat (0, 0.075, 1.25, 2.5, 3.75, and 7.5 ng/mL) were added into the designated wells of a 96-microtiter plate coated with rabbit anti-paraquat antibody. Then, 100 μ L of paraquat-HRP conjugate was added to each well and incubated at room temperature for 30 min. Subsequently, the plate was washed 3 times using washing buffer solution, then blotted for an excess solution. The wells were added with 100 μ L of substrate/color solution, then incubated for another 30 min before 100 μ L of stop solution (3M HCl) was added to each well. The well was measured for absorbance at 450 nm using microplate reader. Standard calibration curves were linear ($r^2 = 0.9929$), limit of detection was 9.76 ng/g dry weight, recovery of extraction was 103.36%, and CV for intra-assay precision ELISA paraquat was 18.80%.

Histological study

The fixed testes were processed using the paraffin method, serially sectioned at 6 μ m, and stained with hematoxylin and eosin (H&E). Histology of all serial sections of the testis was examined under a light microscope and the incidence of TOFs was recorded. The TOFs were indicated by the presence of both testicular and ovarian tissue in the testis, and follicles were found in testicular tissue (Coady et al. 2005).

Data analysis

The overall health of the frog populations was evaluated based on condition factor (CF), as presented by Eastwood and Couture (2002). This indicator of overall health has previously been used in *Fejervarya limnocharis* (Othman et al., 2016). Briefly, the relationship between the logarithm of BW and that of the SVL was determined by regression analysis and is represented in equation (1):

$$\log BW = b \log SVL + \log a \quad (1)$$

Afterwards, the CF was calculated from equation (2):

$$CF = (BW \times 100) / (a \times SVL^b) \quad (2)$$

where constant b is a scaling coefficient or an indicator of the growth pattern of a population.

Gravimetric analysis was used to examine changes in the status of the testis. The gonadosomatic index (GSI) was determined from the weight ratio of the testis to the BW (Othman et al., 2016). The CFs and morphometric parameters of adult, subadult, and juvenile frogs were analyzed using one-way ANOVA, followed by Tukey's HSD post hoc test to identify pairwise differences. Statistical significance was set at $p \leq 0.05$. Additionally, an independent sample t-test was conducted to compare the GSI between adult and subadult frogs.

RESULTS

Health status of the sampled male rice frogs, *F. limnocharis*

The overall health status, in terms of the CF, of male frogs caught from CUFRS showed a mean CF of 103.06 ± 14.35 , 116.88 ± 45.42 , and 93.92 ± 9.44 for adult, subadult, and juvenile groups, respectively (Fig. 2A). There was no significance difference between the three developmental stages ($p > 0.05$).

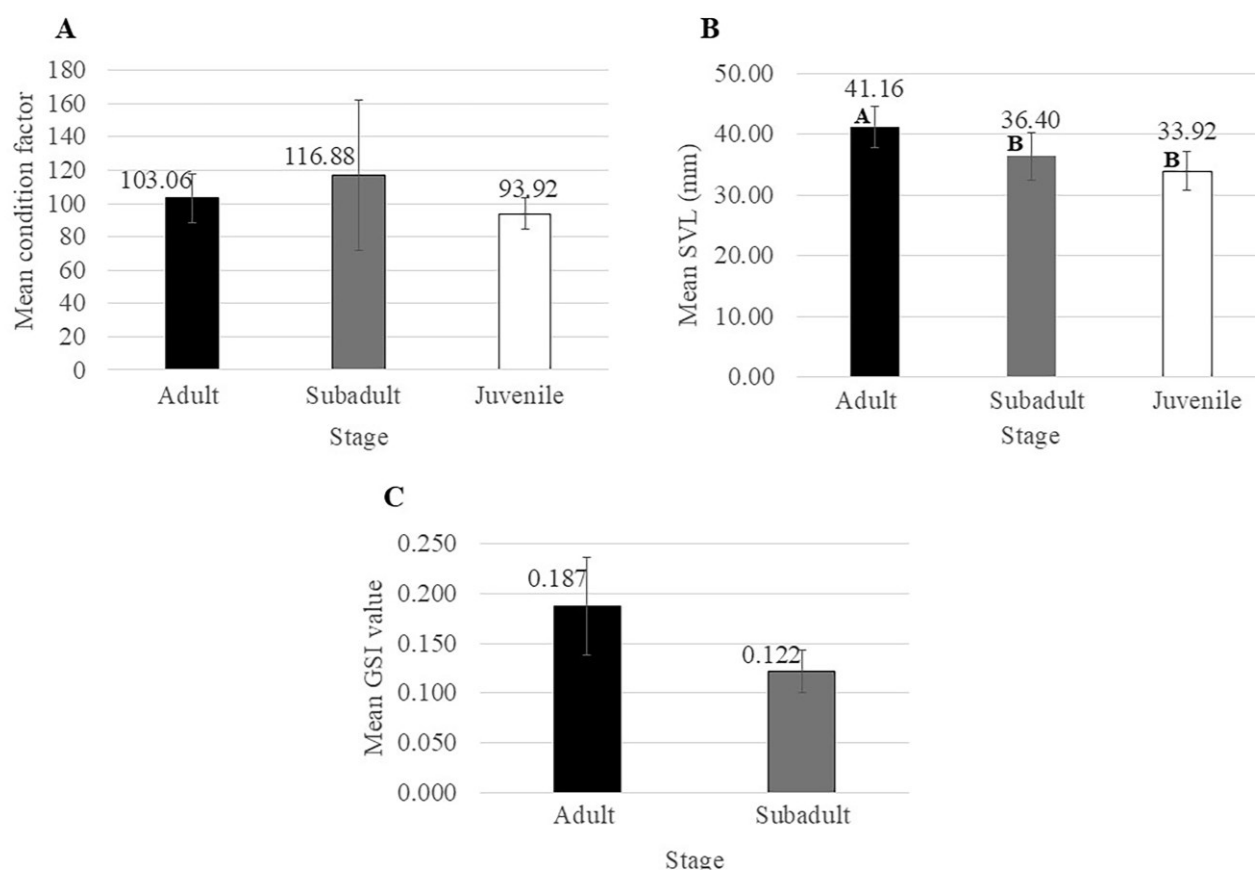


FIGURE 2. Average condition factor (CF; A), snout-vent length (SVL; B), and gonadosomatic index (GSI; C) of *F. limnocharis* living in the Chulalongkorn University Forest and Research Station (CUFRS).

Morphometric and gravimetric analyses of male *F. limnocharis*

The morphometric analyses of male frogs in the adult, subadult, and juvenile groups revealed an average SVL of 41.16 ± 3.45 , 36.40 ± 3.93 , and 33.92 ± 3.20 , respectively (Fig. 2B). The adult group had a significantly higher SVL than the subadult and juvenile groups ($p < 0.001$), but there was no significant difference in the SVL between the subadult and juvenile groups ($p > 0.05$).

The GSI is the weight ratio of testes to the total BW of male frogs, which were divided into the two growth stages of adult and subadults. The GSI of these frogs was 0.122 ± 0.021 in the subadults and 0.187 ± 0.049 in the adult frogs (Fig. 2C), which was not numerically but not significantly different ($p > 0.05$).

Herbicide contamination in frog tissue

Levels of paraquat in every frog sample ($n = 6$) caught from the CUFRS in November 2018 were below limit of detection (9.76 ng/g dry weight) of the Abnova ELISA kit.

Histology of gonad and intersex condition of male *F. limnocharis*

The normal testis in adult male *F. limnocharis*

showed spermatogenesis in seminiferous lobules, which consist of the cysts of spermatogenic cells at the same stage of cell division (Fig. 3). Each cyst develops into spermatozoa and then the sperms are released from the cyst into the lumen of the seminiferous lobules.

Histological study of *F. limnocharis* testicular tissue in an apparent intersex condition (Fig. 4B) showed that the structure of the male reproductive organ was similar to that of a normal testis (Fig. 4A), but oocytes were also found in the testicular tissue. The intersex condition, as indicated by the presence of TOFs, in male frogs living in the CUFRS were investigated in a total of 63 animals (50 adults, seven subadults, and six juveniles). Incidences of TOFs in adult, subadult, and juvenile frogs were 46/50 (92%), 7/7 (100%), and 6/6 (100%), respectively. All the TOFs found in the testis were diplotene oocytes at previtellogenic stage. Furthermore, the characteristics of the oocytes varied. Several normal oocytes were observed, although some exhibited vacuolation and hyperbasophilic cytoplasm (Fig. 5). The incidence of TOFs varied across size classes, differentiated by distinct body weight and snout-vent length measurements (Fig. 6).

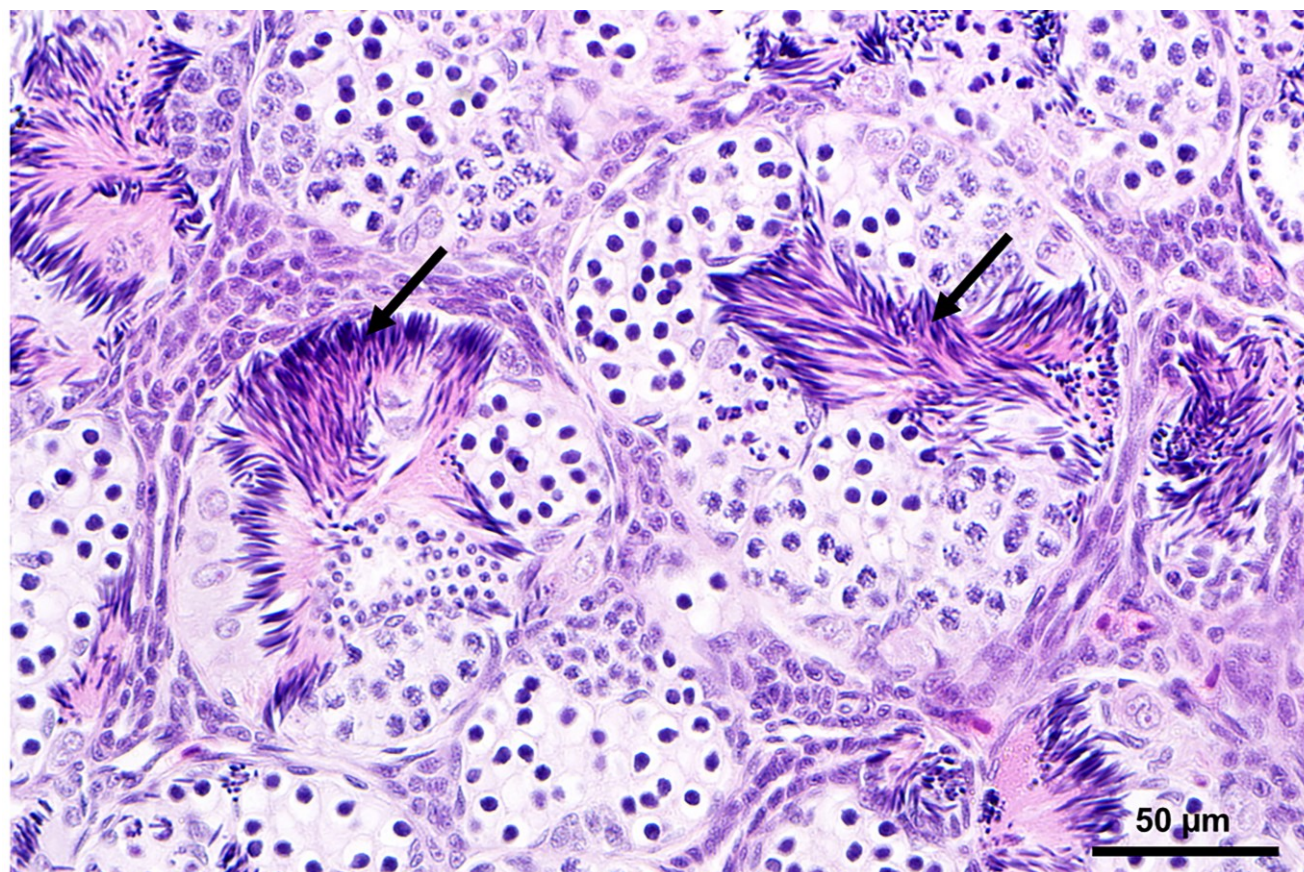


FIGURE 3. Light micrograph of adult testis of *F. limnocharis* with normal development characteristics of spermatogenic cells at the same developmental stage in the cyst. Spermatozoa (arrow) released from the cyst after spermiogenesis.

DISCUSSIONS

The results of this study revealed that male frogs from the herbicide-free CUFRS had similar mean CF values (102.16 ± 11.696) to that reported previously for adult frogs from the herbicide-contaminated Nan province (101.87 ± 1.47) (Thammachoti et al., 2012 and Jantawongsri, 2014). However, the GSI values of the adult stage male frogs differed, being larger (0.23 ± 0.01) in frogs from CUFRS than those in Nan Province (0.17 ± 0.01) (Thammachoti, et al., 2012 and Jantawongsri, 2014), while frogs from Tak Province had a GSI value of 0.1 – 3 (Othman, 2009). In prior studies, the rice frog *F. limnocharis* has been successfully used as a sentinel species to monitor the potential relationship between agricultural activity, in form of herbicide utilization, and altered morphometric and gravimetric parameters (Thammachoti et al., 2012; Othman et al., 2016). However, this current study showed that the CF (an indicative of overall health) and GSI of frogs living in an area with no history of herbicide utilization (CUFRS) in central Thailand were comparable to those in rice frogs living in high agrochemical exposed areas in northern Thailand

(Thammachoti et al., 2012; Jantawongsri, 2014). In this study, the prevalence of TOFs in *F. limnocharis* showed no consistent association with body weight or snout vent length, indicating that TOFs occurrence is not related to frog size.

Histological investigation of the testes showed that the total prevalence rate of TOFs in juvenile, subadult, and adult frogs was over 90%. Since there is no evidence of herbicide utilization in this area (unpublished data), and the paraquat residues in frog tissue were below detection limits, the high incidence of TOFs in *F. limnocharis* may suggest that the intersex gonad may occur as a natural process of testicular development through the semi-differentiated or undifferentiated type of sex differentiation (Gramapurohit et al., 2000; Phuge and Gramapurohit, 2013) in the rice frog. Note that TOFs are transitional structures that can appear during male gonad differentiation. This has also been observed in *Hylarana malabarica*, in which male gonads differentiate through the degradation of the ovarian cavity and the degeneration of oocytes at complete metamorphosis (Mali and Gramapurohit, 2015). In *Rana nigromaculata*, testis-ova differentiation occurs

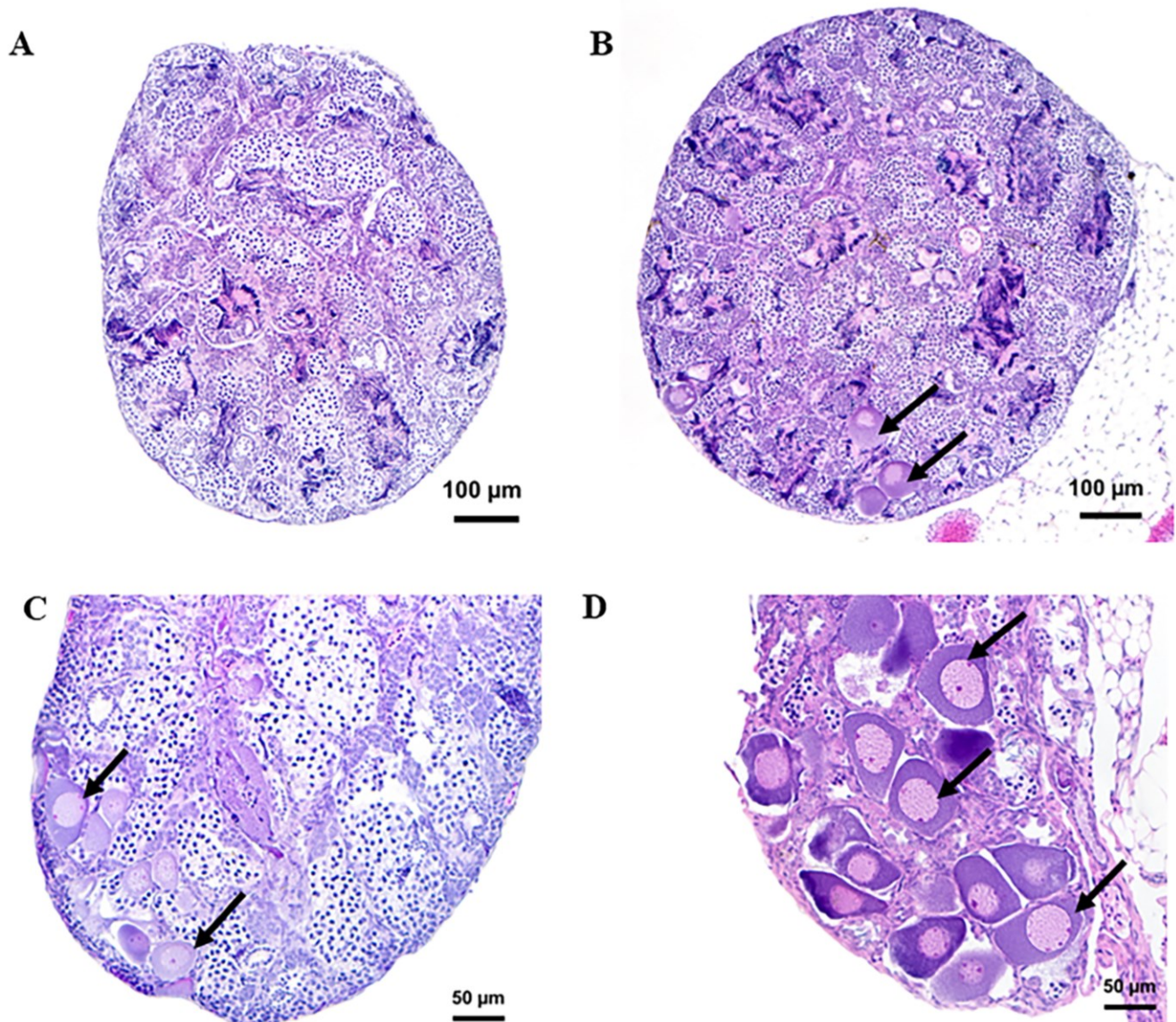


FIGURE 4. Light micrographs of (A) normal testis and (B) testis with TOFs (arrows); TOFs of the (B) adult male, (C) subadult, and (D) juvenile rice frogs (*F. limnocharis*).

during spermatogonial proliferation and is associated with the onset of spermatogenesis. However, the testis-ova were mainly observed in young and subadult males, and not in adult males. Thus, testis-ova formation in *R. nigromaculata* likely occurs during the sexual maturation of subadult frogs at the age of one year (Kobayashi et al., 2015). Sex differentiation in *H. rugulosus* also showed TOFs in males from the juvenile stage through to adult. This indicates that TOFs are present during testicular development through the process of ovarian tissue degeneration in *H. rugulosus*, which exhibits an undifferentiated type of sexual differentiation (Trajitt et al., 2020). In addition, the presence of intersex gonads (testicular oocytes) may occur during certain developmental

periods in frogs and may be correlated with the type of sexual differentiation rate in each species — retarded, basic, and accelerated. Intersex gonads were found to be a normal occurrence during sexual differentiation in *Rana sphenoccephala* (accelerated rate), while in *Bufo americanus* (retarded rate) and *Hyla versicolor* (basic rate), intersex gonads were only found during the juvenile stages (Storrs-Méndez and Semlitsch, 2010). Furthermore, diplotene stage oocytes were found in all TOs of *F. limnocharis* in this study. The diplotene stage represents a prolonged meiotic arrest, during which oocytes become individually enclosed by follicular cells, establishing the basis for ovarian follicle formation (Ogielska et al., 2024).

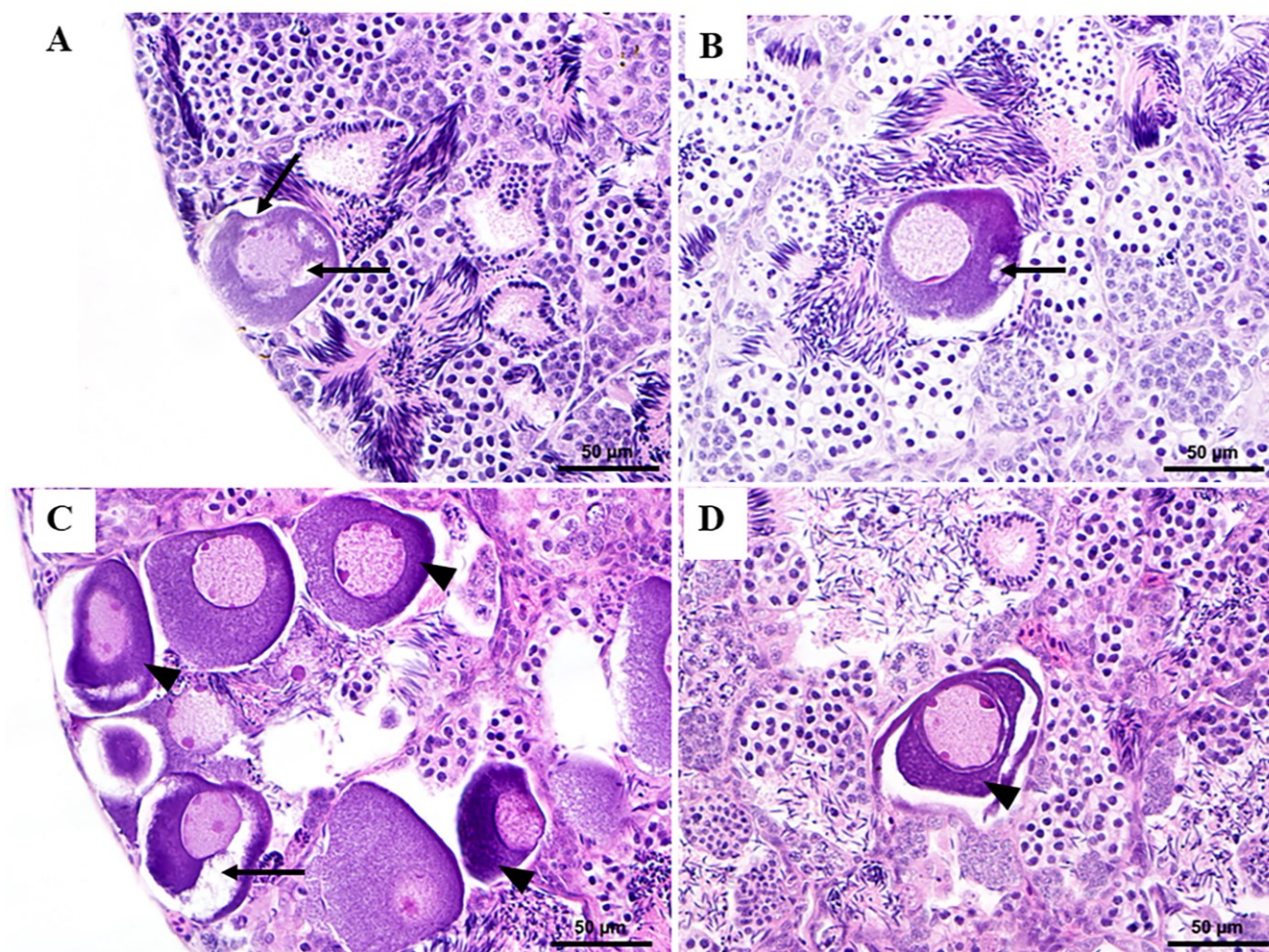


FIGURE 5. Light micrographs of (A – C) atretic TOFs with vacuolation in ooplasm (arrows) and (C, D) hyperbasophilic ooplasm (arrowhead) of *F. limnocharis*.

The frog *F. limnocharis* has been used as a sentinel species for monitoring environmental contamination in several studies in Thailand. One study, conducted in two areas with natural cadmium exposure at different levels in Mae Sot District, Tak Province, found that the *F. limnocharis* population at the contaminated site had higher cadmium concentrations in tissues — including the liver, kidney, testes, and whole body — compared to the reference site. The contaminated site also exhibited a higher macro-melanophage count in the liver and showed occurrences of hepatocyte swelling, renal tumor-like aggregations, and renal hemorrhage. However, TOFs were found in males from both sites, with no significant differences in their frequency in males between the groups (Othman, 2009; Othman et al., 2016). Comparison between two agricultural areas in Nan — one with no history of herbicide use (reference site) and one with herbicide application (contaminated site) — revealed that paraquat

concentrations were significantly higher in frog tissues from the contaminated site. The results indicated a negative impact of herbicide exposure on the overall health of the frogs. Liver and ovarian weights were higher in frogs from the contaminated site, whereas testis weights were not significantly different between the two groups (Thammachoti, et al., 2012). Histological examination of the gonads showed that TOFs were present, but the incidence of TOFs was not significantly different between the contaminated and reference sites (Kitana et al., 2018). A subsequent study by Jantawongsri (2014) found that frogs from the two areas exhibited significantly different immune responses, further supporting the impact of herbicide exposure on frog health. Based on the analysis of several organ tissues and their physiology, *F. limnocharis* has been suggested as a potential sentinel species in ecotoxicity research. However, using TOs as a biomarker might not be suitable in this species, as our

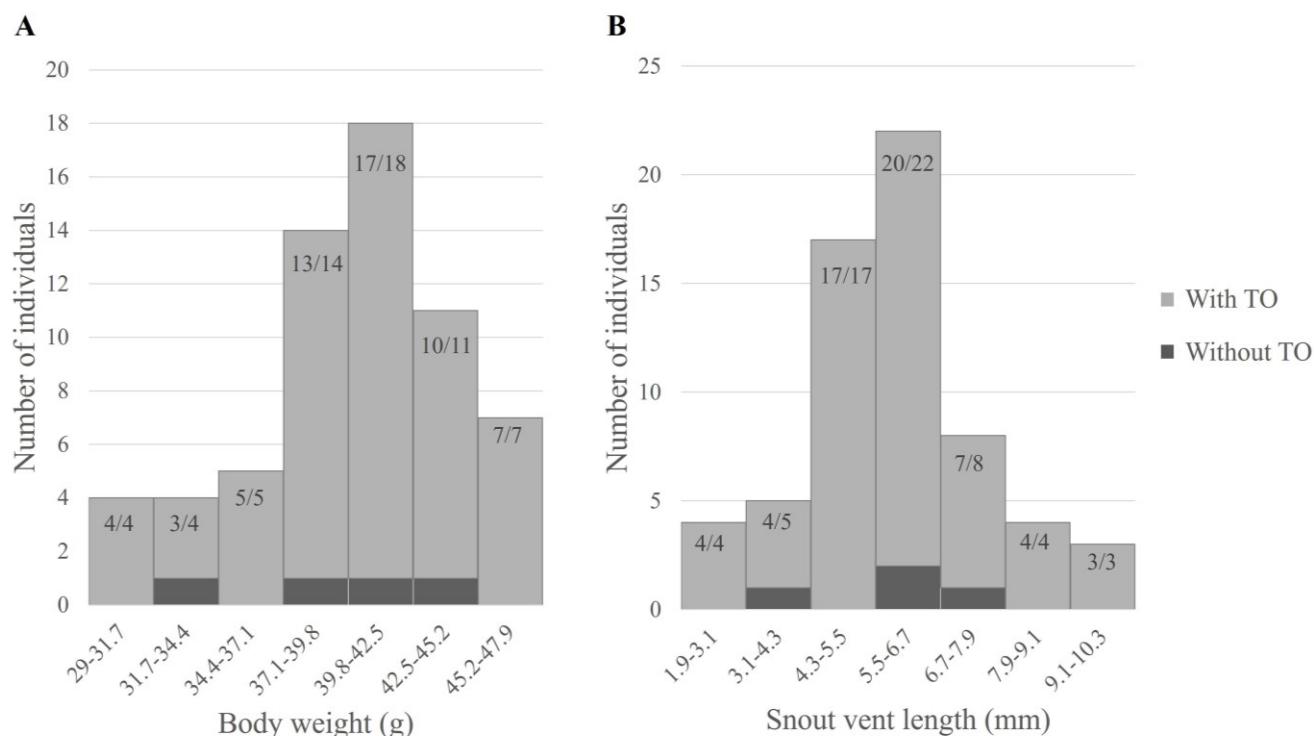


FIGURE 6. Histogram showing the number of male *F. limnocharis* with and without TOs in different size class: body weight (A) and snout vent length (B).

study found that TOs were present in all developmental stages of male *F. limnocharis*, even in areas with no history of herbicide use. As TOs were known to be used as a biomarker for contamination by herbicides (e.g., atrazine, glyphosate, and paraquat), heavy metals (e.g., cadmium) and insecticides (e.g., DDT) that act as EDCs, our results therefore suggest that TOs in *F. limnocharis* may be a natural process during gonadal differentiation. Therefore, we recommend that using TOs as a biomarker of effect in this species should be carefully interpreted. Further studies are required to confirm whether the intersex condition in the *F. limnocharis* results from low-level contamination of endocrine disrupting chemicals in their habitat or as a natural process of gonadal development. Accordingly, investigating gonadal development under controlled laboratory conditions is essential. If the latter is confirmed, the presence of TO should not be used as a valid biomarker of effect in *F. limnocharis*.

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