

Immunoreactivities of AR, ER α , ER β and aromatase in the nuptial pad of Chinese brown frog (*Rana dybowskii*) during pre-hibernation and the breeding period

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ABSTRACT

There is a prominent local raised pad called nuptial pad on the forelimb of Chinese brown frog (*Rana dybowskii*), which is hypothetically concluded as an enhancement of the grip and a spreader of pheromone during the amplexus. In this study, we investigated the immunolocalization and protein expression levels of androgen receptors (AR), estrogen receptor α (ER α), ER β and aromatase in the nuptial pad of *R. dybowskii* during pre-hibernation and the breeding period. Histologically, the annual development of the nuptial pad in *R. dybowskii* is manifested as the larger area of specialized mucous gland and the longer length of papillary epidermal projection during the breeding period. AR, ER α , ER β and aromatase are present in the stratum granulosum, stratum spinosum, stratum basale and the secretory portion of specialized mucous glands during both periods. Western blotting results confirmed that AR, ER α and ER β protein levels are higher during pre-hibernation than those during the breeding season. These results suggest that nuptial pad is the direct target organ of androgen and estrogen. Androgen may participate in the regulation of annual development and glandular function of nuptial pad, and estrogen may play an endocrine, autocrine or paracrine role during pre-hibernation and the breeding period.

Key words: Androgen receptor; aromatase; estrogen receptor; nuptial pad; *Rana dybowskii*.

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Introduction

The nuptial pad is a secondary sexual character of male anurans,¹ and the presence or structural details of nuptial pad are commonly included as standard information in taxonomic descriptions. Nuptial pads are modified areas of skin located at least on the medial margin of finger II of most male anurans, whose invariable characteristic is the presence of sexually dimorphic skin glands (SDSGs) in the dermis, and during the breeding period, nuptial pads develop annually manifesting as thickened epidermis or dermis, or both, when compared with adjacent skin.² In the nuptial pads of most species, these SDSGs correspond to specialized mucous glands (SMGs) that consist of a secretory portion, a neck and an intraepidermal duct, which allows molecules synthesized and stored in the SMGs to be channeled to the pad's surface during amplexus and may play an important role in pheromone secretion during courtship.^{2,3} Moreover, further study indicated that amplexins are secreted at papillary epidermal projections (PEPs) of the male nuptial pad which only secrete during the breeding period, the structures of which show similarities with the plethodontid modulating factors (PMF), and the results demonstrate the possible existence of an ancient pheromone system in amphibians.⁴ Interestingly, testosterone could deal several regulating effects on the nuptial pad including increases in thickness of both the epidermis and dermis, formation of PEPs, and development of SMGs.⁵

The regulation of androgens on the reproduction of amphibians is manifested in many aspects, including the development of secondary sexual character, the synthesis and delivery of pheromone, the formation of reproductive behavior, *etc.*^{6,7} Androgen such as testosterone and dihydrotestosterone (DHT) are mainly synthesized by the testis and they are effective in two main ways: directly activate androgen receptors (AR) or conversion into estradiol activate certain estrogen receptors (ER).^{8,9} Sex steroids are indispensable for the production of male chemical signals related to mating in red-legged salamander. Levels of plasma testosterone are positively associated with development of the male mental gland which secretes courtship pheromones during the breeding season.¹⁰ Testosterone together with prolactin (PRL) can mediate production of the male courtship chemical signal sodefrin in the cloacal gland of male red-bellied newts which has androgen receptors (ARs) and prolactin receptors (PRLRs). Treatment with testosterone and PRL results in the development of the cloacal gland with increased sodefrin mRNA expression and sodefrin content, and testosterone is required for PRL action.^{11,12} Similarly in male terrestrial salamanders (*Plethodon shermani*), elevated plasma androgen increased expression of a chemo-investigatory behavior (nose tapping), preference for some female-derived chemosensory cues and development of cirri, morphological structures thought to enhance the transfer of chemosensory cues from the substrate to the vomeronasal organ (VNO).¹³ Moreover, androgens are proven to be required for the expression of male mating behavior in dusky salamanders (genus *Desmognathus*).¹⁴

Estrogen is produced by the conversion of androgen in the presence of aromatase, and it also plays an important role on regulating reproduction in amphibians.⁹ The Bidder's organ (BO) is the main source of estrogens in male toads, and it expresses different steroidogenic enzymes as well as aromatase in *Rhinella arenarum*.¹⁵ Estradiol produced by the BO regulates spermatogenesis by increasing apoptosis of cysts in late stages, and also steroidogenesis by reducing testicular 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD/I) activity during the post reproductive season. Furthermore, the ER β is detected not only in the Leydig cells and cysts in the testis, but also in follicular cells and oocytes in the BO, which suggest the possibility that estradiol may act as an autocrine/paracrine role on bidderian steroidogenesis and oogene-

sis.⁹ Besides, in the *Xenopus*, expression level of aromatase is remarkably higher in the brain than in other tissues during the early stages of brain morphogenesis. While aromatase expression level in the brain is increasing, estradiol (E2) in the whole body begins to increase at the same stage. Iwabuchi *et al.* supposed E2 synthesized by the high aromatase expression in the choroid plexus, which generates cerebrospinal fluid, may circulate to the heart and act through ER α during sex differentiation.¹⁶

The Chinese brown frog (*R. dybowskii*) is a special amphibian, whose oviduct expands prior to hibernation instead of expanding during the breeding period, and inhabits the northeastern of China (provinces Heilongjiang, Liaoning and Jilin).¹⁷ *R. dybowskii* hibernates from October to February and then come with reproduction from March to June affected by the latitude and altitude. The nuptial pads of *R. dybowskii* are mainly composed of at least two thickened skins at the base of the first finger.¹⁸ Previous research has found that the morphological and microstructure changes of the nuptial pads of *R. chensinensis* which shares close phylogenetic relationships with *R. dybowskii*, are regulated by testosterone, and change during the annual reproductive cycle.¹⁹ There are many hypotheses on the functions of the nuptial pads during amplexus, such as pheromone delivery through skin abrasion and providing male frogs with better grip,^{1,4,20,21} but more evidence is still needed to prove those assumptions.² The exact function of the nuptial pad in *R. dybowskii* during the amplexus is unknown, neither the role that androgen and estrogen play in the regulation of seasonal morphological change. We suppose the nuptial pads of *R. dybowskii* develop periodically during pre-hibernation and the breeding period, and androgen and estrogen may participate in the seasonal regulation. To extend our understanding of the regulation of nuptial pad thickened in *R. dybowskii*, the aim of the present study is to investigate immunoreactivities of AR, ER α , ER β and aromatase proteins during pre-hibernation and the breeding period, to elucidate the relationship between sex steroid hormones and annual development and glandular function of nuptial pad.

Materials and Methods

Animals

Fifty adult female Chinese brown frogs were obtained in April (n=25, the breeding period), and October (n=25, pre-hibernation), 2019 from Jilin Paektu Mountain Chinese Brown Frog Breeding Farm, Jilin Province, China. All animals were treated in accordance with the National Animal Welfare Legislation. All experimental procedures were conducted by the guidelines established by the Beijing Forestry University. Frogs were anesthetized by diethyl ether. Each nuptial pad was collected from *R. dybowskii*. For every nuptial pad of each frog, half of the nuptial pad was cut perpendicular to the epidermis on the thickest part, and was immediately fixed for 24 h in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in 0.05 M PBS, pH 7.4 for histological and immunohistochemical analysis, with another half of it stored at -80°C for protein extraction.

Histology

The nuptial pad samples were dehydrated in ethanol series and embedded in paraffin. Serial sections (5 μ m) were mounted on slides coated with poly-L-lysine (Sigma Chemical Co.). Some sections were stained with hematoxylin and eosin (HE) for histological observations. We randomly chose 6 sections respectively from 6 frogs during pre-hibernation and the breeding period which were cut perpendicular to the epidermis on the thickest part of the nuptial pad, and used ImageJ software (ImageJ bundled with 64-bit

Java 1.8.0_172) to calculate the area of every SMG and the length of every PEP.

Immunohistochemistry

After being deparaffinized and rehydrated, the nuptial pad sections were incubated with 10% normal goat serum to reduce background staining caused by the secondary antibody. And then the sections were incubated with primary rabbit anti-human polyclonal antibody (1:500 dilution) against AR (sc-7305) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ER α (MC-20) (Santa Cruz Biotechnology), ER β (H-150) (Santa Cruz Biotechnology), and aromatase (H-300) (Santa Cruz Biotechnology) for 12 h at 4°C. The negative control sections were treated with normal rabbit serum instead of the primary antibody. Then the sections were incubated with second antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtraAvidin staining kit (Sigma Chemical Co.), followed by visualizing with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 ml of 0.05 mol Tris-HCl buffer, plus 30 μ l H₂O₂. Finally, the sections for aromatase were counterstained with hematoxylin solution (Merck, Tokyo, Japan). The specificity of aromatase and ER antibodies in this amphibian was previously confirmed.¹⁷ The immunostained slides were examined using a photomicroscope (BX51, Olympus, Tokyo, Japan). The immunohistochemical staining was determined as positive (+), strong positive (++), very strong positive (+++), and negative (-). Staining that was weak but higher than control was set as positive (+); the highest intensity staining was set as very strong positive (+++); staining intensity between + and +++ was set as strong positive (++)

Western blot

The nuptial pads were diced into small pieces with a clean razor blade respectively and then homogenized in a homogenizer containing 300 μ l of 10 mg/ml phenylmethanesulfonyl fluoride (PMSF) stock and incubated on ice for 30 min while maintaining the temperature at 4°C throughout all the procedures. Nuptial pad homogenates were centrifuged at 12,000 \times g for 6 min at 4°C. Protein extracts (25 μ g) were blended with an equal volume of 2 \times Laemmli sample buffer. Equal amount of each sample was loaded and run on a 12% SDS-PAGE gel at 18 V/cm, and then transferred to nitrocellulose membranes using a wet transblotting apparatus for 20 min (Bio-Rad, Richmond, CA, USA). The membranes were blocked in 3% BSA for an hour at room temperature. Primary incubation of the membranes was carried out using a 1:500 dilution of rabbit anti-human AR, ER α , ER β and aromatase antibody, which were the same as used in immunohistochemistry for 12 h and the specificity of aromatase and ER antibodies was also confirmed before.¹⁷ Secondary incubation of the membranes was then carried out using a 1:1000 dilution of goat anti-rabbit IgG for AR, ER α , ER β and aromatase, tagged with horse-radish peroxidase for an hour. The membrane was then colored with 10 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 50 mL phosphate buffer (0.03 M) plus 3 μ l H₂O₂. β -actin was used for the endogenous control. The intensities of the bands were quantified using ImageJ software (ImageJ bundled with 64-bit Java 1.8.0_172).

Statistical analysis

The data were expressed as mean \pm standard error of the mean (SEM). The normality and homoscedasticity tests were evaluated by D'Agostino & Pearson test and F test, respectively. And statis-

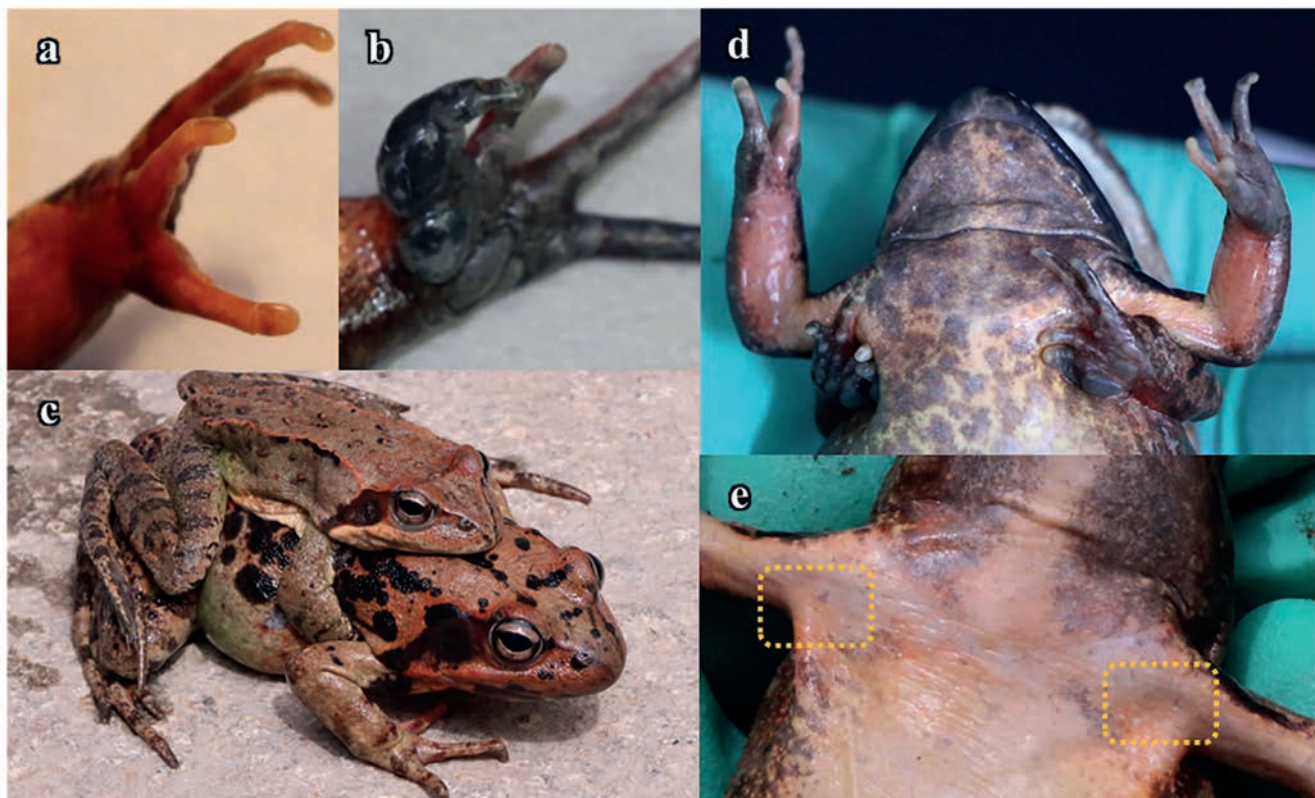


Figure 1. Morphology observation of male and female *R. dybowskii* during the breeding period. a) The finger of female *R. dybowskii* during the breeding period. b) The finger of male *R. dybowskii* with nuptial pads during the breeding period. c,d) The posture of amplexus in *Rana dybowskii*. e) The chest of female *R. dybowskii* after amplexus.

tical significance between differences groups was ensured by two-tailed unpaired Student's *t*-test by GraphPad Prism 7. A value of $p < 0.05$ was considered indication of statistical significance.

Results

Morphology

During the breeding period, compared with the finger of female frog (Figure 1a), nuptial pads appearing as thickened skins were clearly identified at the base of the Finger I and extended to Fingers II and III (Figure 1b). Figure 1 c,d shows the posture of amplexus in *R. dybowskii* that the male clasps the female about the back and puts its hands closely to the axillar/pectoral area of the female frog. According to our observation, there was no visible wound on the corresponding position of chest (Figure 1e), same with the other *R. dybowskii* we obtained. Figure 2 a,b shows the appearance of the nuptial pads of *R. dybowskii* during the breeding period, and Figure 2 c,d shows the nuptial pads during pre-hibernation, which cover the entire pre-axial part of the thumb in both periods.

Histology

The nuptial pads of *R. dybowskii* consist of several layers of cells, including the stratum corneum (SC), the stratum granulosum (SG), the stratum spinosum (SS), the stratum basale (SB), the stratum spongiosum (SSP) and the stratum compactum (SCO) (Figure 3). One layer of SC, one layer of SG, one to several layers of SS,

and one layer of SB form the epidermis from outside to inside, while SSP and SCO form the dermis part. There are PEPs on the nuptial pads, which are formed by an epidermal and dermal evagination (Figure 3 a,b). Melanophores locate in the stratum spongiosum of the dermis (Figure 3 a-d). The morphology of SMGs is alveolar, and SMGs are formed by an intraepidermal duct, a neck and a secretory portion, most of which are located in SSP (Figure 3 b,c,e,f). Compared with pre-hibernation (Figure 3 d,e,f), the area of SMG and the length of PEP during the breeding period are significantly larger and longer (Figure 3 a-c), as shown in Figure 4.

Immunohistochemistry

Immunolocalizations for AR, aromatase, ER α and ER β in the nuptial pads of *R. dybowskii* during pre-hibernation and the breeding period are present in Figure 5. Compared with negative control sections (Figure 5 i,j,k,l), AR is specifically localized in the SG, SS, SB and the secretory portion of SMGs during pre-hibernation and the breeding period, and during pre-hibernation the positive signal is stronger (Figure 5 a,b). Similarly, aromatase, ER α and ER β are found in the SG, SS, SB and the secretory portion of SMGs during the breeding period, and are also positioned in the SG, SS, SB and the secretory portion of SMGs during pre-hibernation with a stronger positive stain by ER α and ER β (Figure 5 c-h). The negative control sections (Figure 5 k and l) were stained with hematoxylin, while Figure 5 i and j were not. The staining results obtained from the images are quantified and summarized in Table 1.

Western blot

The protein levels of AR, aromatase, ER α and ER β in the nup-

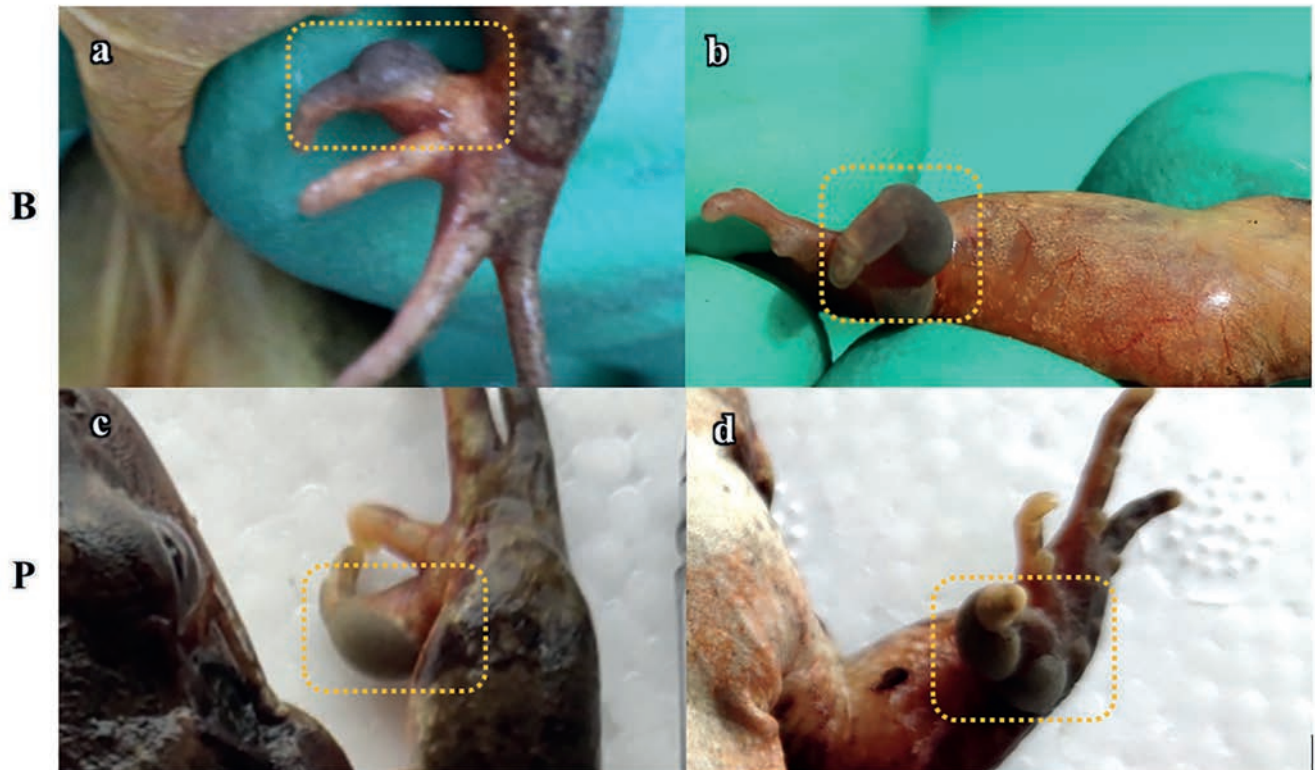


Figure 2. Morphology observation of male *R. dybowskii* during pre-hibernation and the breeding period. a b) The male *R. dybowskii* with nuptial pads during breeding period. c,d) The male *R. dybowskii* with nuptial pads during pre-hibernation. B, the breeding period; P, pre-hibernation.

tial pad during pre-hibernation and the breeding period were examined by Western blotting, and the results were shown in Figure 6. The positive bands for AR, aromatase, ER α and ER β were detected at a molecular weight of 110 kDa, 66k Da, 56 kDa and 55 kDa respectively (Figure 6 a- d). The protein levels of AR, aromatase, ER α and ER β in the nuptial pad of pre-hibernation are higher than those of the breeding period and the protein levels of AR, ER α and ER β show significant difference. The quantification results were normalized to the expression level of β -actin.

Discussion

In the present study, we reported the immunoreactivities of AR, ER α , ER β and aromatase in the nuptial pad of *R. dybowskii*. The histological results show that the area of SMG and the length of PEP during the breeding period are significantly larger and longer than those during pre-hibernation. The immunohistochemical data demonstrate that AR, ER α , ER β and aromatase are present

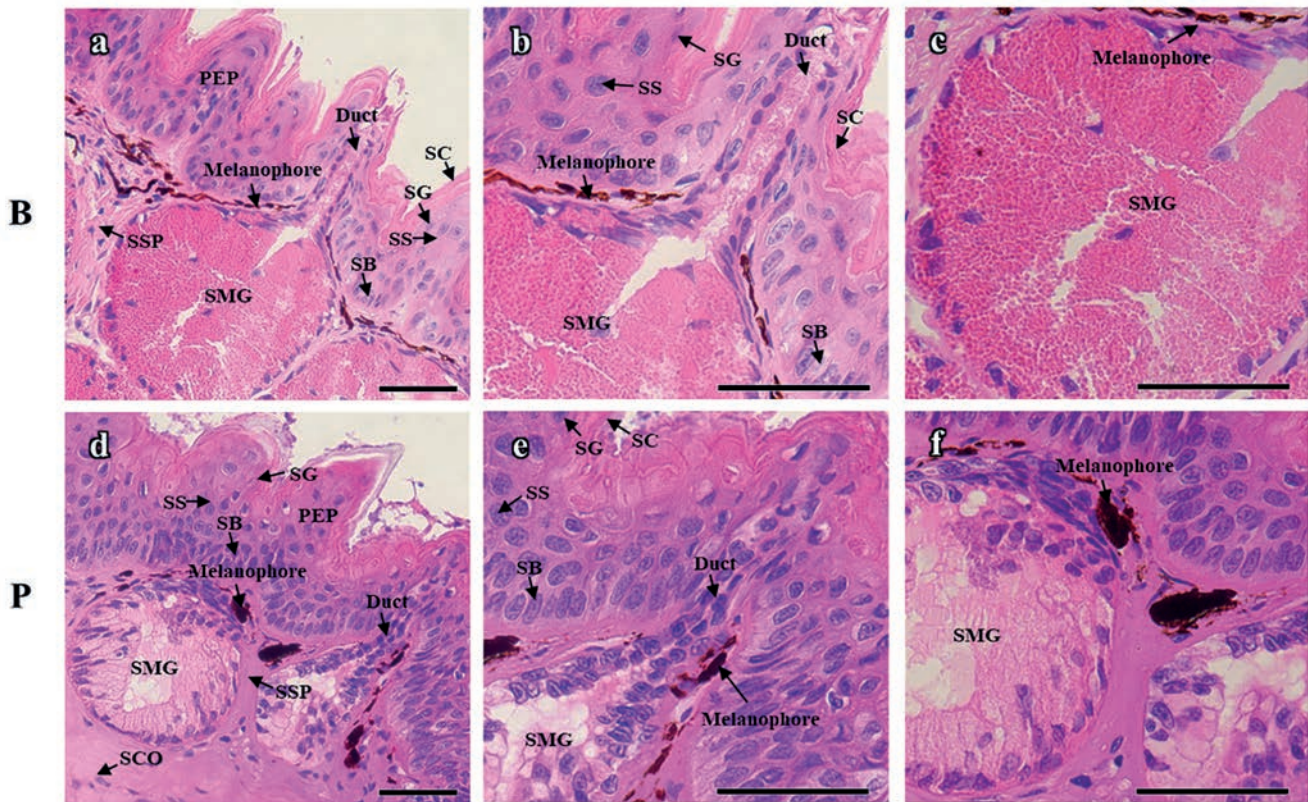


Figure 3. Histological structure of *R. dybowskii* nuptial pad by hematoxylin-eosin (H&E) during pre-hibernation and the breeding period. a) The nuptial pad during breeding period. b,c) Partial enlarged views of a. d) The nuptial pad during pre-hibernation. e,f) Partial enlarged views of d. SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; PEP, papillary epidermal projection; SMG, specialized mucous gland; SSP, stratum spongiosum; SCO, stratum compactum; B, the breeding period; P, pre-hibernation. Scale bars: 50 μ m.

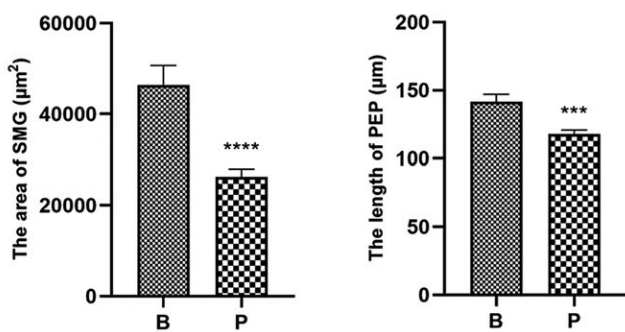


Figure 4. Measurement result for the area of SMG and the length of PEP during pre-hibernation and the breeding period. Data were shown as the mean \pm SEM (n=3, each period). ***p<0.001; ****p<0.0001; B, the breeding season; P, pre-hibernation.

Table 1. Immunohistochemical localizations of AR, aromatase, ER α and ER β in the nuptial pad of *R. dybowskii* during pre-hibernation and the breeding period.

Antibody	SG		SS		SB		SMG	
	B	P	B	P	B	P	B	P
AR	+	++	+	+++	++	+++	+	++
Aromatase	+++	+	+++	++	+++	+++	++	++
ER α	++	++	++	++	++	++	+	+++
ER β	+	++	+	++	+	+++	+	++
NC	-	-	-	-	-	-	-	-

-, negative staining; +, positive staining; ++, strong positive staining; +++, very strong positive staining; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; SMG, specialized mucous gland.

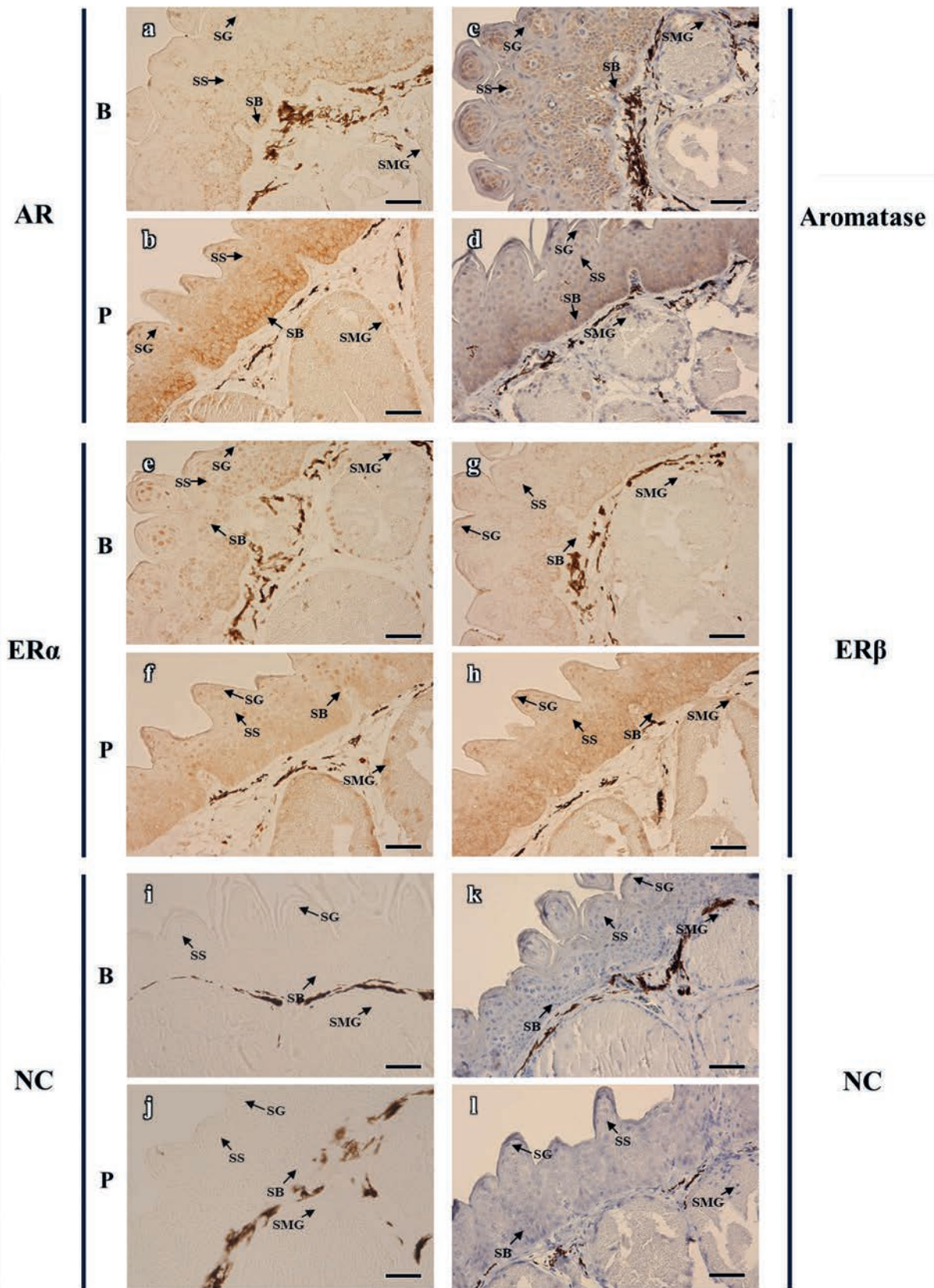


Figure 5. Immunolocalizations for AR (a,b), aromatase (c,d), ER α (e,f) and ER β (g,h) in the nuptial pads of *R. dybowskii* during pre-hibernation and the breeding period. Black arrows indicate the positive signal of AR, aromatase, ER α and ER β in the SG, SS, SB and the secretory portion of SMGs during pre-hibernation and the breeding period. h, i, j, k: Negative control. k and l were stained with hematoxylin. SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; SMG, specialized mucous gland; NC, negative control; B, the breeding period; P, pre-hibernation. Scale bars: 50 μ m.

in the SG, SS, SB and the secretory portion of SMGs during both periods. The protein levels of AR, ER α , ER β and aromatase are higher during pre-hibernation than those during the breeding period, and the protein levels of AR, ER α and ER β in two periods show significant difference. These results suggest that androgen may participate in the regulation of annual development and glandular function of nuptial pad, and estrogen may also play an important role *via* an endocrine, autocrine or paracrine manner during pre-hibernation and the breeding period.

Histologically the nuptial pads of *R. dybowskii* during the breeding season are significantly increased in size, which are manifested as the larger area of SMG and the longer length of PEP, and the results are similar to other researches about the nuptial pad of anurans. For instance, the researches about the annual patterns of nuptial pad in the male Chinese bullfrog (*R. rugulosa*) find that nuptial pad epidermis and mucous glands develop rapidly in January and February, and during the breeding season (March-July), the well-developed nuptial pad is rough, papillate and thick;

mucous glands are hypertrophied and lumen secretions are abundant.²² The researches in *R. ridibunda* also support these results.²³ Luna *et al.* studied the morphological variation of the nuptial pads in 26 species of *Phyllomedusinae* (Amphibia: Anura: Hylidae), and confirmed the existence of intraepidermal duct in SMG in the nuptial pad of most species, which is consistent with our findings.²⁴ It might contribute to the spread of secretions on the nuptial pad and other surfaces like the axillar/pectoral area of female frog during amplexus. Furthermore, Epstein *et al.* found that the nuptial pads of male *R. pipiens* treated with androgen showed strong morphological evidence of activity. For example, the dermal and epidermal layers were thick, the PEPs were developed and the glands exhibited large acini lined with columnar epithelial cells with well-defined cellular boundaries.²⁵ Moreover, Willaert *et al.* also found the nuptial pad of *R. temporaria* cause wounds on the female's chest during amplexus and hypothesized that the secreted molecules can seep directly into the female's circulatory system.⁴ But according to our results, there is no macroscopical wound on the

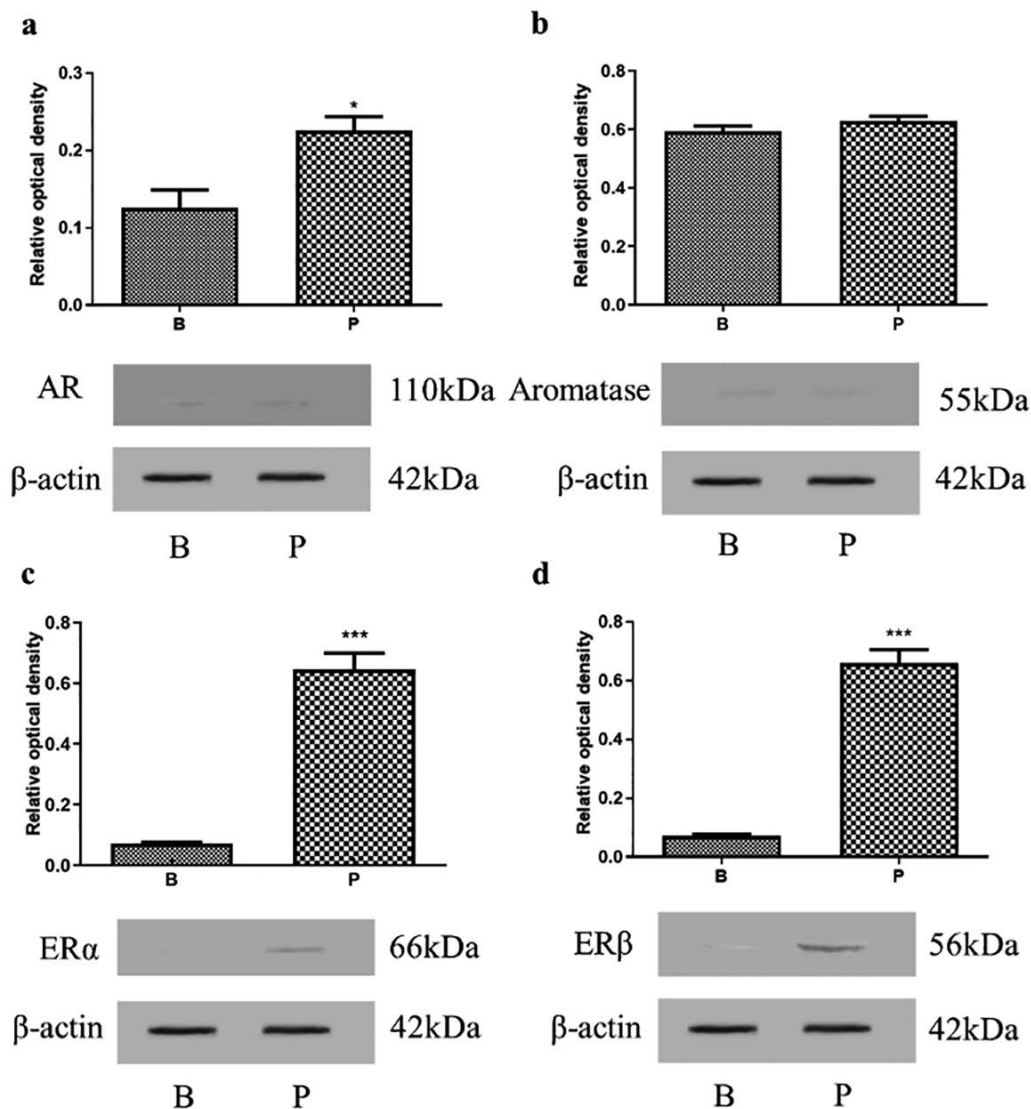


Figure 6. Western blotting analysis for AR(a), aromatase(b), ER α (c) and ER β (d) in nuptial pads during pre-hibernation and the breeding period. Data were shown as the mean \pm SEM (n = 6, each period, 4 independent experiments). *p<0.05, ***p<0.001, B, the breeding season; P, pre-hibernation.

chest of female *R. dybowskii* after amplexus, this hypothesis still needs further evidence.

Gonadal steroid hormones especially androgen and estrogen are important in development and seasonal variation of sexually dimorphic organs, regulating the synthesis and release of pheromone, and then influencing the reproductive behaviors.^{6,26} Our results demonstrate that AR, ER α , ER β and aromatase are present in the SG, SS, SB and the secretory portion of SMGs during both periods. Previous researches indicate that treatment with androgen, or up-regulation of plasma androgen levels, can promote the development of the nuptial pad, such as darkness, SMG size, and other histological characteristics,^{5,22,23,27} which suggest the nuptial pad is the target organ of androgen. Furthermore, Orton *et al.* found nuptial pad features (size/color/number of hooks/androgen receptor mRNA) are positively associated with amplexus success, and demonstrated the central role for nuptial pad morphology in reproductive success in *Xenopus tropicalis*.²⁸ Our Western blot results show that the protein levels of AR, ER α , ER β and aromatase are higher during pre-hibernation than those during the breeding period, and the protein levels of AR, ER α and ER β in two periods show significant difference. Fasano *et al.* studied the intratesticular feedback mechanisms in the regulation of steroid profiles in the *R. esculenta*, and concluded that testosterone shows high values during early spring (between February and March) and autumn (between October and November), with a rapid decrease thereafter.²⁹ Considering the time, we obtained *R. dybowskii* was April and October, our protein level results that AR expressed significantly higher in pre-hibernation than that in the breeding period consistent greatly with previous researches. Moreover, Delgado *et al.*³⁰ found that the environmental temperature and photoperiod seasonally play an important role in the regulation of the annual testicular cycle in *R. perezi*, depending on the phase of the annual reproductive cycle. For example, during pre-breeding period, a shortening in photoperiod causes a reduction in testosterone, while low temperature increases testosterone plasma levels during the post-breeding period.³⁰ Further researches are still needed to explore the mechanism and physiological significance of this regulation in *R. dybowskii*.

The expression of aromatase is an indication of estrogen production, since the conversion of androgens to estrogen by aromatase is the rate-limiting step of estrogen biosynthesis.³¹ Most genes have estrogen response elements, and estrogen autocrine/paracrine regulation of local tissue or organ structure and function is common with widespread estrogen receptor.^{32,33} Fasano *et al.* studied sites of action of local estradiol feedback mechanism in the testis of *R. esculenta* and concluded that estradiol inhibits androgen synthesis by decreasing the activity of steroidogenic enzymes starting from 17 α -hydroxylase.³⁴ More interestingly, van Wyk *et al.* assess the estrogenic contaminants disruption to androgenic control system in male *Xenopus laevis* which works mainly through anti-androgenic activity, by using the histology of the androgen-dependent SMG in nuptial pad.³⁵ Besides, in a similar structure, Zhang *et al.* concluded that estrogen-related genes may be the main factors regulating the seasonal development of the keratinized nuptial spines on upper jaw in *Leptobranchium boringii*, by assessing the *de novo* transcriptome using brain, testis and upper jaw skin and comparing gene expression profiles of these tissues between two critical periods of the spine growth cycle.³⁶ These researches and our results that ER α , ER β and aromatase share similar immunolocalizations in the nuptial pad of *R. dybowskii* suggest that estrogens may participate in the regulation of annual development and glandular function of nuptial pad *via* an endocrine, autocrine or paracrine manner during pre-hibernation and the breeding period. According to our protein level results that there is

no difference in the expression level of aromatase in the two periods, we assume there is also no difference in the level of estrogen. The elevated ER protein level improves the sensitivity to estrogen and increases regulation function of estrogen. Another possible explanation is that post-translational mechanisms may be involved in regulating estrogen synthesis in the nuptial pad of *R. dybowskii*, according to the study in the hypothalamus of the female *R. esculenta*.³⁷

In conclusion, the annual development of the nuptial pad in *R. dybowskii* is manifested as the larger area of SMG and the longer length of PEP during the breeding period. Nuptial pad is the direct target organ of androgens and estrogens. Androgens may regulate the annual development and glandular function of nuptial pad, and estrogens may participate *via* an endocrine, autocrine or paracrine manner during pre-hibernation and the breeding period. However, the exact function of nuptial pads and PEPs in male is still unclear, neither the origin of the skin modifications. Also, further researches are needed to figure out chemical composition of secretions in nuptial pad SMGs and their roles in reproduction.

Acknowledgments

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