# ผลของความเค็มต่อการเปลี่ยนแปลงระดับการแสดงออกของโปรตีนตัวรับไทรอยด์ฮอร์โมนชนิดแอลฟาในลูกอ๊อดกบนา ชนิด *Hoplobatrachus rugulosus*

# Effect of Salinity on the Alteration of Thyroid Hormone Receptor Alpha (TRα) Protein Expression in the Chinese edible Frog, *Hoplobatrachus rugulosus*, Tadpole

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**บทคัดย่อ**

ไทรอยด์ฮอร์โมนมีบทบาทหน้าที่เกี่ยวข้องกับการเจริญเติบโตและการเปลี่ยนแปลงรูปร่างของสัตว์จำพวกกบ ซึ่งพบว่าน้ำเค็มส่งผลต่อการเจริญเติบโตและการเปลี่ยนแปลงรูปร่างของลูกอ๊อดชนิดต่างๆ ดังนั้นงานวิจัยในครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลกระทบจากความเค็มต่อระดับความเข้มข้นของไทรอยด์ฮอร์โมนและระดับการแสดงออกของโปรตีนตัวรับไทรอยด์ฮอร์โมนชนิดแอลฟาในเหงือก ผิวหนัง และครีบหางของลูกอ๊อดกบนาในช่วงระยะโพรเมทามอร์โฟซิส โดยเริ่มต้นเลี้ยงลูกอ๊อดกบนา (*Hoplobatrachus rugulosus*) ระยะที่ 27–30 ในสารละลายเกลือโซเดียมคลอไรด์เข้มข้น 0 2 4 และ 6 ส่วนในพันส่วน เป็นเวลา 21 วัน พบว่าระดับไทรอยด์ฮอร์โมนเพิ่มขึ้นเมื่อเลี้ยงลูกอ๊อดในน้ำเกลือเข้มข้น 2 และ 4 ส่วนในพันส่วนเมื่อเทียบกับกลุ่มควบคุม แต่ในกลุ่มลูกอ๊อดที่เลี้ยงในน้ำเกลือเข้มข้น 6 ส่วนในพันส่วน ไม่เปลี่ยนแปลง การแสดงออกของโปรตีนตัวรับไทรอยด์ฮอร์โมนชนิดแอลฟาพบว่าในเนื้อเยื่อเหงือก ผิวหนัง และหาง มีขนาด 34 กิโลดาลตัล การแสดงออกของโปรตีนตัวรับฮอร์โมนไทรอยด์ชนิดแอลฟาในเนื้อเยื่อเหงือกและผิวหนังเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเลี้ยงลูกอ๊อดในสารละลายเกลือเข้มข้น 2 และ 4 ส่วนในพันส่วน ในขณะที่ลดลงอย่างมีนัยสำคัญเมื่อเลี้ยงลูกอ๊อดในสารละลายเกลือเข้มข้น 6 ส่วนในพันส่วน เมื่อเทียบกับกลุ่มควบคุม นอกจากนี้ยังพบว่าระดับการแสดงออกของโปรตีนตัวรับไทรอยด์ฮอร์โมนชนิดแอลฟาในเนื้อเยื่อครีบหางลดลงอย่างมีนัยสำคัญเมื่อเลี้ยงลูกอ๊อดในสารละลายเกลือเข้มข้น 2 ส่วนในพันส่วน ในขณะที่ไม่มีการเปลี่ยนแปลงเมื่อเลี้ยงในสารละลายเกลือเข้มข้น 4 และ 6 ส่วนในพันส่วน จากผลการทดลอง

สามารถสรุปได้ว่า สารละลายเกลือที่มีความเข้มข้นต่ำกว่าหรือเท่ากับ 4 ส่วนในพันส่วนจะส่งผลในการเพิ่มระดับไทรอยด์ฮอร์โมนและเพิ่มระดับการแสดงออกของโปรตีนตัวรับไทรอยด์ฮอร์โมนชนิดแอลฟาซึ่งอาจจะเป็นเหตุผลหลักที่ทำให้มีการเพิ่มการเจริญเติบโตของลูกอ๊อดกบนา


## ABSTRACT

Thyroid hormone (TH) promotes growth and remodels of the tadpole into complete adult frog. Since water salinity is a one factor effect on metamorphosis of anuran. Thus, the present study aims to study the effects of saline water on the levels of total body fluid of TH ($T_4$) and TR**α** protein expression in gill, skin, and tail fin of *Hoplobatrachus rugulosus* tadpoles at pro-metamorphic stage. Tadpoles (stage 27–30) were exposed to salinities (NaCl); 0, 2, 4 and 6 parts per thousand (ppt) for 21 days. The total body fluid of $T_4$ increased in 2 and 4 ppt groups, while in 6 ppt group was not change. Western blot analysis revealed that 34 kDa of TR**α** was detected in the larval gill, skin and tail fin. The expression of TR**α** protein increased in gill and skin in 2 and 4 ppt groups, but decreased in 6 ppt group. Decreased of TR**α** protein expression in tail fin was found in tadpoles exposed to 2 ppt, but not in 4 and 6 ppt groups. These suggest that at low water salinity (≤4 ppt) increased in $T_4$ and TR**α** protein could be involved in promoting growth and metamorphosis in *H. rugulosus* tadpoles.


**คำสำคัญ:** เกลือโซเดียมคลอไรด์ ไทรอยด์ฮอร์โมน โปรตีนตัวรับไทรอยด์ฮอร์โมน การเปลี่ยนแปลงรูปร่าง
**Keywords:** NaCl, Thyroid hormone, Thyroid hormone receptor protein, Metamorphosis

## INTRODUCTION

Amphibian metamorphosis is complex developmental processes that change almost every tissue and organ of the tadpole (Dodd and Dodd, 1976; Kikuyama et al., 1993; Shi et al., 1996), which these changes controlled by thyroid hormone (TH). TH induces morphological changes such as, cell death and tissue resorption of tail and gill and remodeling of tadpole organs, i.e., intestine, epidermis, and muscle (Dodd and Dodd, 1976). TH regulates gene expression by way of the TH receptors (TR). The cDNA cloning of TR in mammals and chickens (Sap et al., 1986; Weinberger et al., 1986) identified them as members of the nuclear receptor superfamily of transcription factors. There are two isoforms of TR, TR**α** and TR**β**, and both are highly similar, 85% similarity (Yaoita and Brown, 1990).

The TR**α** mRNA accumulates throughout the pre-metamorphosis stage and it is optimum at the stage of pro-metamorphosis, while the TR**β** mRNA level

increases in synchrony with the endogenous TH concentration which rises to a peak at the climax of metamorphosis, suggesting distinct functions for TRα and TRβ (Yaoita and Brown, 1990). The wide expression of TRα has been reported in different amphibians' species (Banker et al., 1991; Kawahara et al., 1991). During metamorphosis, the high level of TRα was present in various tissues (Tata and Widnell, 1966; Oppenheimer et al 1995). Many factors influence the TR expression (Krain and Denver, 2004). Reported in *Bufo calamita* tadpoles which were exposed to high salinity showed delayed metamorphosis and coincided with TH level decreasing (Gomez-Mestre et al., 2004).

Metamorphosis retardation caused by many environmental factors, i.e., low food levels, extreme temperature, and low pH (Ultsch et al. 1999). Water salinity is a stressor that has been less studied to date, although it is likely to be important, since most amphibians avoid brackish waters. The salinization of wetland and freshwater ecosystem often results from climate change such as rising of sea levels (Nielsen et al., 2003; Hsu et al., 2012) as well as anthropogenic processes (Williams, 2001; Nielsen and Brock, 2009). Amphibians are generally sensitive to osmotic stress (Uchiyama and Yoshizawa, 1992; Haramura, 2007). Several reports have present that salinity causes negative effects on survival, development, metamorphosis and behavior of tadpoles (Chinathamby et al., 2006; Wu and Kam 2009; Squires et al., 2010; Nakkrasae et al., 2015).

A Chinese edible frog, *Hoplobatrachus rugulosus*, is an economically important species. It is widely distributed in southeast Asia and southern China (Khonsue and Thirakhupt, 2001). In Thailand, the population of this species is also present in saline wetland with salinity levels ranging from 2–4 ppt (Suwannatrai et al., 2011). Previous study found that *H. rugulosus* tadpoles reared in salinity of 6 ppt reduced their size, delayed metamorphosis and could not develop to juvenile frog (Nakkrasae et al., 2015). Therefore, we hypothesize that salinity might effect on level of thyroxine ($T_4$) and the expression of TRα.

The present study thus aims to study the effects of saline waters on the level of $T_4$ and expression of TRα protein in gill, skin, and tail fin of *H. rugulosus* tadpole at pro-metamorphic stage.

## RESEARCH METHODOLOGY

### 1. Animals and experimental design

Larvae of *H. rugulosus* stage 27–30 (Gosner, 1960; Figure 1A) were obtained from a commercial farm in Maha Sarakham Province, Thailand. They were kept at the Aquaculture Laboratory, Khon Kaen University,

Thailand. They were maintained in a glass tank containing aerated dechlorinated tap water (15 L) with salinity of 0 ppt as determined by a refractometer. Then they were randomly cultured in four salinity levels (n = 50 per group, i.e., 0 , 2, 4 and 6 ppt, adjusted by dissolving NaCl (99.99% purity without iodine; Univar, New South Wales, Australia) in tap water. The larvae were cultured in 15 L water in a 23.5 × 49.5 × 30 $cm^3$ glass tank. Half of the water was replaced every 3 days with tap water with designated salinity. Tadpoles were fed daily with 5% of total body weight of frog chow, containing 42% wt/wt protein and 3% wt/wt lipid ad libitum. The experiment was maintained under natural light (12 h dark: 12 h light). During the 21-day experimental period, tadpole stages were checked every day until the tadpoles developed to stage 38–40 (Gosner, 1960; Figure 1B). The gill, skin and tail fin of tadpoles were then sampled for determining TR**α** protein expression. This study has been approved by the Animal Ethics Committee of Khon Kaen University, Thailand (record no. AEKKU 6/2557; reference no. 0514.1.12.2/6).

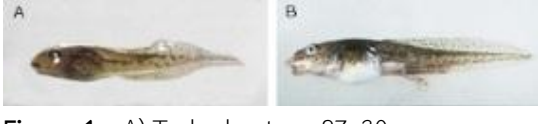


**Figure 1** A) Tadpole stage 27–30.
B) Tadpole stage 38–40.

**2. Measurement of $T_4$ levels**

Hormone concentrations in total body fluid were determined. At the stage 38–40, tadpoles were sacrificed after an ice-induced anesthetization. Then, the tadpoles were ground to a fine powder in liquid nitrogen, and homogenized. After centrifugation at 12,000 *g* for 15 min, the supernatant was collected as total body fluid. Determination of $T_4$ was performed by radioimmunoassay kit (catalog no. 07BC-1007; MP Biomedicals, NY, USA). Standards and samples were added into $T_4$ antibody-coated tubes. Anti-rabbit $T_4$ was coated onto the inner surface of polypropylene tubes. [$^{125}$I] $T_4$ was added to all tubes, followed by incubation at 37 °C for 60 min, and then washed and desiccated the tubes. The coated tubes were determined for the amount of [$^{125}$I] $T_4$ radioactivity by using a gamma counter (PerkinElmer, MA, USA). The detection range of this kit was 0–250 ng/ml.

**3. Protein extraction and western blot analysis**

Tissues of *H. rugulosus* tadpoles were isolated. Protein were extracted from gill, skin and tail fin of *H. rugulosus* tadpoles using lysis buffer (0.5 mM Tris, pH 7.5, 1.5 M NaCl, 10 mM Na-ethylene- diaminetetra acetic acid, 10% TritonX-100, 10% sodium dodecyl sulfate (SDS), 1 mM phenylmethyl-sulfonyl fluoride (PMSF). Protein concentrations were

determined in triplicate by Nanodrop spectrophotometer (Maestrogen, Las Vegas, USA). Protein (50 μg) was separated by 12% SDS-polyacrylamide (SDS-PAGE) gel electrophoresis. Following electrophoresis, proteins were transferred to 0.45 μm a polyvinylidene difluoride membrane (PVDF; UltraCruz®, Santa Cruz Biotechnology, Texas, USA). Membrane was blocked for 1 h at 4 ºC in blocking buffer containing 150 mM NaCl, 10 mM Tris, pH 7.6, 0.1% Tween-20 and 1% (w/v) bovine serum albumin (Vivantis, CA, USA). Subsequently, the membranes were incubated with anti-TRα or anti-β actin antibodies (Abcam, Massachusetts, USA) at 1:1000 dilution at 4 ºC overnight. Anti-TRα antibody was raised in rabbits using a human TRα. Then membranes were incubated with HRP conjugated goat anti-rabbit secondary antibody at 1:5,000 dilutions (Abcam) at 4 ºC overnight. The signal of protein expression was detected with a chemiluminescent substrate (ECL; Pierce, Thermo Scientific Rockford, USA), and subsequently exposed to autoradiography film.

**4. Statistical analysis**

All results were presented as means ± SEM and data were analyzed by GraphPad Prism 6.0 (San Diego, CA, USA). One-way analysis of variance (ANOVA) was used for analysis of differences between groups with Dunnett's posttest. The level of significance for all statistical analyses was 95% confidence interval ($P < 0.05$). Molecular weight and quantification of protein expression were analyzed by UVI 1D software (UVITEC Cambridge, Cambridge, UK).

**RESULTS**

Salinity affected the amount of $T_4$ as shown in Figure 2 ($F = 10.75$, $df = 3$, $P = 0.001$). The amount of $T_4$ in total body fluid was higher in 2 and 4 ppt saltwater groups than in the control group. On the other hand, $T_4$ level in 6 ppt saltwater group was not different from control group (Figure 2).

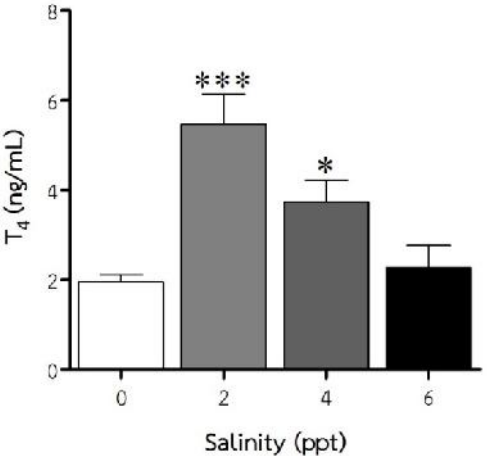


**Figure 2** Amount of thyroid hormone ($T_4$) in total body fluid of *H. rugulosus* tadpoles at Gosner stage 38–40 reared in three water salinity treatments; 0, 2, 4 and 6 ppt. Data are mean ± SE (n = 8). *$P < 0.05$, ***$P < 0.001$ compared with control 0 ppt (one-way ANOVA).

Western blot analysis revealed that 34 kDa of TRα was detected in the larval gill (Figure 3), skin and tail fin. Changes in the expression of TRα protein in gills, skin, and tail fin were investigated after salt water exposure. TRα in gill and skin was significantly difference among salinity groups ($F$ = 124.50, $df$ = 3, $P$ < 0.0001, Figure 4; $F$ = 234.10, $df$ = 3; $P$ < 0.0001; Figure 5, respectively).

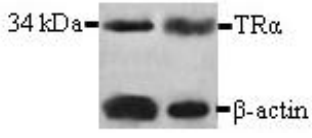


**Figure 3** Western blot analysis of TRα protein expression. Protein (50 μg) from gill of *H. rugulosus* tadpole stage 38–40 was used. Each lane represents each individual tadpole. β-actin was used as an internal control.

The protein expression of TRα was found to increase in gill and skin after transfer of tadpole from fresh water to 2 and 4 ppt salt water as compared with the expression of 0 ppt group (Figures 4 and 5). While, decreasing level of TRα protein expression of both tissues was found in 6 ppt group. The expression of TRα proteins in tail fin of *H. rugulosus* tadpole was significantly difference among salinity groups ($F$ = 17.11, $df$ = 3; $P$ < 0.05). Decrease of TRα protein expression in tail fin was found in tadpoles exposed to 2 ppt, but not in 4 and 6 ppt salinities (Figure 6).

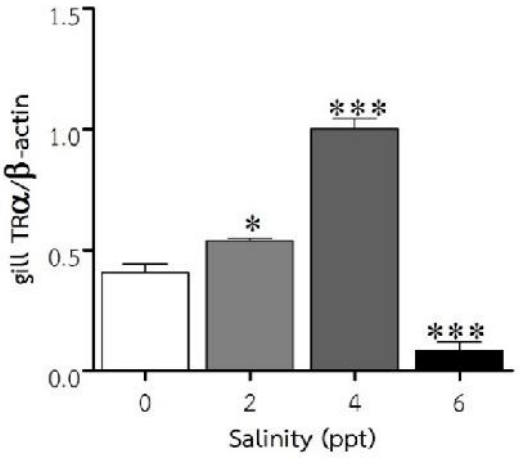


**Figure 4** Effects of salt waters on gill protein expression of TRα of *H. rugulosus* tadpoles stage 38–40. Values are expressed as means ± SE (n = 8). *$P$ < 0.05, ***$P$ < 0.001 compared with control 0 ppt.

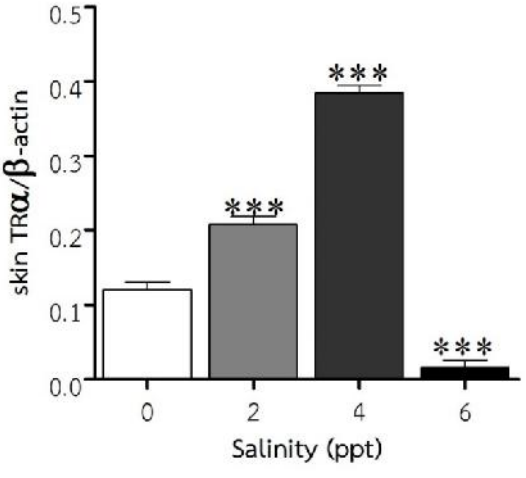


**Figure 5** Effects of salt waters on skin protein expression of TRα of *H. rugulosus* tadpoles stage 38–40. Values are expressed as means ± SE ($n$ = 8). ***$P$ < 0.001 compared with control 0 ppt.

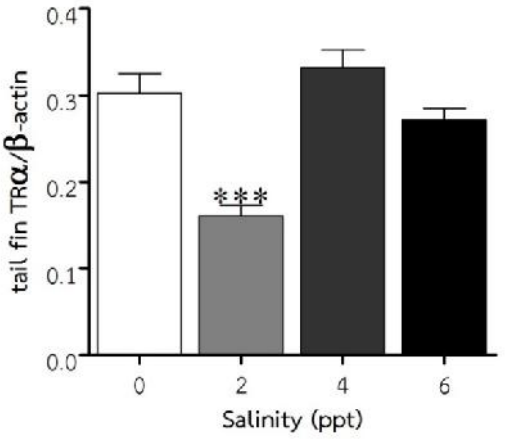


**Figure 6** Effects of salt waters on tail fin protein expression of TRα of *H. rugulosus* tadpoles stage 38–40. Values are expressed as means ± SE ($n$ = 8). ***$P$ < 0.001 compared with control 0 ppt.

## DISCUSSION AND CONCLUSION

A major characteristic of metamorphosis, in amphibians, is that each organ undergoes different patterns of programmed morphogenetic and functional changes in direct response to the metamorphic hormonal signal (Gosner, 1960). Thyroid hormone controls diverse processes of morphogenesis, such as regression of tail and gill and extensive morphological and biochemical remodeling of the skin. In the present study we could detect the level of $T_4$ and expression of TRα protein in gill, skin and tail fin of *H. rugulosus* during late pro-metamorphic stage (stage 38-40, Gosner, 1960). Previously reports in *Xenopus laevis* was also found TRα mRNA in the same developmental stage (Yaoita and Brown, 1990). There is an apparent surge in the production of TH at metamorphic climax in *Rana catesbeina* (White and Nicoll, 1981). However, in *X. laevis*, the level of $T_4$ rises gradually from late pre-metamorphic stages to a peak at midclimax (Buscaglia et al., 1985).

Our previous report showed that at low salinity levels (< 4 ppt) increased both growth rates and developmental of *H. rugulosus* tadpoles, while high salinity at 6 ppt reduced on metamorphosis (Nakkrasae et al., 2015). Sped and delayed metamorphosis due to water salinity might involve $T_4$ which were increased in low salinity and decreased in high salinity (Figure 1). We propose two possible mechanisms of salinity-induced decrease in thyroid hormone levels worth further research. First, the production of prolactin since it is well known as osmoregulatory hormone in aquatic animals (Manzon, 2002) could be increased under saline conditions and antagonize the thyroid hormone. Since prolactin is known to antagonize the thyroid hormone effect on growth and meta-morphosis of tadpoles (White and Nicoll, 1981; Denver, 1996; Shi et al., 1996). Second, glucocorticoids such as corticosterone are under the same pituitary control as mineralocorticoids, which in turn are important in osmoregulation. Corticosterone

has been shown to delay metamorphosis when increased during early development in *Bufo boreas* (Hayes, 1995) and could be upregulated under saline conditions.

Several investigators have analyzed the expression of TRα in tadpoles during metamorphosis (Denver, 1998). In *X. laevis* TRα expression increases shortly after hatching and is maintained at a high level throughout metamorphosis climax (Shi et al., 1996). TRα transcripts are detectable in all tadpole cells that have been examined (Berry et al., 1998). Therefore, study the expression of TRα in *H. rugulosus* in the present study was chose to study in late-prometamorphic stage. During this stage, cell proliferation increased in the skin (Furlow and Neff, 2006; Schreiber et al., 2001), which correlated to the levels of TH and TRα protein of *H. rugulosus* already increased (Figures 2 and 5). This suggested that TH might involve in remodeling of skin in *H. rugulosus.* However, TRα was expressed in resorbing tissues such as the gill and tail fin, which at this time undergoes extensive cell apoptosis (Denver, 1998). During pro-metamorphic stage, gill begun to resorb which related to increase in $T_4$ and TRα protein in *H. rugulosus* tadpole. While tail fin were shorten later in metamorphic climax, this might be the reason why low detection of the TRα protein in the tail fin. The salinity affected on TRα protein expression which might be indirectly controlled by $T_4$. The pattern of TRα protein expression responded in osmotic stress between gill and skin is relatively to TH level, while that of in tail fin showed decreased of the receptor in 2 ppt group. These demonstrated that the hormone acts directly on individual tissue to produce the diversity of developmental switching.

In summary, *H. rugulosus* tadpoles is very sensitive to environmental condition because of their habitat and complex processes of metamorphosis regulated by the endocrine system, mainly TH. In the present study, the tadpoles were exposed to low water salinity (≤4 ppt) increased in TH and TRα protein concentrations, presumably causing increased developmental rate (Nakkrasae et al., 2015). While, lower TH and TRα protein concentrations found in tadpoles in high water salinity (6 ppt) which might cause in reducing developmental rate.

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