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## Sex Maturation Induction for Snakeskin Gourami (*Trichopodus pectoralis*) during Off-Season Spawning by a Long-Photoperiod and a High Water Temperature Manipulation

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The present study was carried out to induce a gonadal maturation of snakeskin gourami (*Trichopodus pectoralis*) by a long-photoperiod and a high water temperature manipulation during an off-season spawning. A natural photoperiod and ambient water temperature conditions (NC; 11.5L:12.5D, 24.8±0.3°C) was compared with an artificial long-photoperiod and a high water temperature (ALP+AT; 18L:6D, 28.4±0.1°C) in a 60 days experimental period (5 January to 20 March 2017). Plasma 17β-estradiol (E<sub>2</sub>), gonado-somatic index (GSI), and percentage of the final oocyte maturation (FOM) from the ALP+AT were significantly higher than that of the NC on day 30<sup>th</sup> and day 60<sup>th</sup> after exposure. GSI (10.26±0.36%) obtained from this study is almost the same value with a reported GSI of snakeskin gourami females in the spawning season (June to October). A full oocyte maturation (FOM) was observed at 34.7±5.3% from fish exposed to ALP+AT, but it was not detected in the NC group. After the 60 days exposure, only fishes exposed to ALP+AT were successfully induced by Buserelin acetate (30 µg·kg<sup>-1</sup>) combined with Domperidone (10 mg·kg<sup>-1</sup>) to spawn and they were consequently fertilized rendering 1,442±338 hatched embryos·spawner<sup>-1</sup>.

**Keywords:** Ovary, Photoperiod, Reproduction, Snakeskin gourami, Temperature

### INTRODUCTION

An air-breather gourami fish, snakeskin gourami (*Trichopodus pectoralis* Regan, 1910), is native to the Mekong and Chao Phraya basin of Thailand, Cambodia, Vietnam, and Laos. It has been widely used for aquaculture in many countries include Malaysia, Indonesia, Philippines, Myanmar, Sri Lanka, Papua New Guinea, New Caledonia and Thailand (Food and Agriculture Organization [FAO], 1988). In Thailand, it is among the top five economically important freshwater fish (Department of Fisheries, 2019). Conventionally, snakeskin gourami is reared in a swamp pond modified from a rice field. In this system, the male

and female broodstocks are stocked in a late summer from April to May each year to allow a bubble nesting and later spawned between June and September in the same year. Hatched embryos are still reared in the same pond to approximately eight to ten months to marketable size (100 to 200 g) and they are finally harvested between March and April of the following year. Thus, its production is seasonal. Although previous studies have been carried out to using a synthetic hormone-induced spawning technique to increases production (Amornsakun et al. 2004; Rukdontri, 2010), the technique requires only a fully matured broodstock based on seasonal developmental.

This leaves the off-season spawning for a potential snakeskin gourami marginal production.

It is generally known that seasonal changes in photoperiod and water temperature are the important environmental cues in stimulating gametogenesis in fish which activate through the hypothalamus-pituitary-gonadal axis (HPG axis) (Falcon et al. 2007; 2010). Thus, the induction of gonadal maturation using a long-photoperiod and a high water temperature had been demonstrated in many tropical fishes include Nile tilapia (*Oreochromis niloticus*) (Biswas et al. 2005; El-Sayed and Kawanna, 2007), Indian major carp as *Catla catla* (Dey et al. 2004; 2005), *Labeo rohita* and *Cirrihinus mrigala* (Sarkar et al. 2010), blue gourami (*Trichogaster trichopterus*) (Levy et al. 2011), goldfish (*Carassius auratus*) (Sarkar and Upadhyay, 2011), and catfish (*Clarias batrachus*) (Acharia et al. 2014). A long-photoperiod (13L:11D) manipulation stimulates gonadal activity in female catfish (*C. batrachus*) resulted in higher plasma  $17\beta$ -estradiol ( $E_2$ ) level and gonado-somatic index (GSI) increased higher than that reared under a natural photoperiod (11L:13D) (Acharia et al. 2014). In terms of water temperature, female blue gourami (*T. trichopterus*) reared in high water temperature (27°C) rendered a significant rise of the final oocyte maturation (FOM) percentage (Levy et al. 2011). These results confirm the influences of environmental cues on fish gonadal development. In addition, a combination of long-photoperiod (15.5L:8.5D) and high water temperature (27-29°C) regimen effectively induced a gonadal maturation of the Indian major carp (*C. catla*, *L. rohita*, and *C. mrigala*) gonads. As a result, induced spawning was successfully carried out at least four to five months earlier than their normal spawning season (Sarkar et al. 2010). The above-mentioned demonstrated that a long-photoperiod and a high water temperature manipulation can be used to induce maturation on off-season spawning in various species of tropical fishes. Evidently, there is a possibility of using the mentioned manipulation for fishes. Due to a diversity of fish species include their gonadal development patterns, the application should be confirmed to each species. The off-season spawning is an alternative approach for the snakeskin gourami marginal production regardless of a rearing area expansion. To our knowledge, there was no evidence of using a long-photoperiod and a water temperature manipulation in inducing an ovarian maturation of snakeskin gourami female during an off-season spawning.

Thus, the present study is the first attempt to

apply the artificial long-photoperiod and high water temperature manipulation to induce an ovarian maturation of snakeskin gourami during an off-season spawning. Knowledge gained is important information for alternative practices to broodstock management and seed production of snakeskin gourami during the off-season spawning.

## MATERIALS AND METHODS

### Fish and experimental conditions

Immature Snakeskin gourami (*Trichopodus pectoralis*) 20 males and 40 females with an average mean length of  $10.29\pm 0.14$  cm and  $10.94\pm 0.16$  cm respectively were collected from a local fish farm in Khon Kaen province, Thailand, since December 2016. The male and female fish were kept separately and maintained under ambient photoperiod and water temperature in a fiberglass tank (500 L) inside the hatchery building at the Department of Fishery, Khon Kaen University, Thailand (16°28'N 102°49'E) and acclimatized for 15 days before an experiment begins (5 to 19 January 2017).

At the beginning of the experiment (20 January 2017), blood samples were drawn from a caudal vein of five random females using EDTA coated syringe [0.5 M EDTA (pH 8.0)] and centrifuged at 10,000 rpm under 4°C for 10 min. Then, plasma was collected and stored at -20°C until further  $17\beta$ -estradiol ( $E_2$ ) assay. After decapitation, ovaries were removed and weighed. Gonado-somatic index (GSI) was calculated according to the following formula:  $GSI = [\text{gonad weight (g)} / \text{body weight (g)}] \times 100$ . In addition, ovaries were fixed in 10% formalin solution. Then, the remaining fish were randomly allocated into the aerated experimental tanks (50 L; 55 × 40 × 30 cm<sup>3</sup>) includes four tanks for males and six tanks for females, with a rearing density of 1 fish·10 L<sup>-1</sup> (5 fish·tank<sup>-1</sup>).

Two treatments using photoperiod and water temperature manipulation were set up include natural photoperiod and ambient water temperature conditions (NC; 11.5L:12.5D,  $24.8\pm 0.3^\circ\text{C}$ ) and artificial long-photoperiod combined with artificial high water temperature (ALP+AT; 18L:6D light on at 3:00 AM,  $28.4\pm 0.1^\circ\text{C}$ ). Ten males and fifteen females were allocated in each treatment. For ALP+AT treatment, the artificial light was provided using 28 watt fluorescent lamps (daylight: 6500 k) with 1,000 lx light intensity at the water surface. The experimental units were covered with a black polyethylene sheet to prevent ambient light

interference. The assigned artificial high water temperature was supplied with a 200 watt heater set at 28°C.

During a 60 days experimental period (20 January to 20 March 2017), fish were fed to satiation with a commercial fish diet ( $\geq 40\%$  crude protein,  $\geq 4\%$  fat) at 10% body weight one time daily at 12:00 PM. The tanks were cleaned and replaced with fresh water at 50% every three days to maintain a hygienic condition that remained within the optimum level required for freshwater fish rearing dissolved oxygen  $>3 \text{ mg}\cdot\text{L}^{-1}$  and pH 6.5-9. The temperature daily recorded at 6:00 AM and 6:00 PM.

Sampling for the estimation of plasma  $E_2$ , GSI, and the ovary histological examination on the female fish was done twice in a 60 days experimental period. The first sampling was done on day 30<sup>th</sup> of the experiment (19 February 2017) where one of three female tanks was taken from each experimental group. The second sampling was done on day 60<sup>th</sup> of the experiment (20 March 2017) where three of ten female fish in the remaining two female tanks from each experimental group. The samples were used for the mentioned three reproductive parameters again.

The remaining six females and six males from each experimental group were subjected to a hormone-induced spawning using Buserelin acetate ( $30 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ ) combined with Domperidone ( $10 \text{ mg}\cdot\text{kg}^{-1}$ ) as described by Rukdontri (2010). Observed parameters include egg production, hatching rate, and fertility which were evaluated at 48 h after hormone injection.

In this study, both males and females were simultaneously exposed to the same photoperiod and water temperature manipulation. The gonadal development in the females only was examined throughout the experiment because its sensitivity to the mentioned manipulation is higher than that of the males. However, the males were used for final a fertilization.

#### **Plasma $17\beta$ -estradiol ( $E_2$ ) assay**

Plasma  $E_2$  was estimated by enzyme immunoassay (EIA) conducted at the Laboratory of hormonal analysis in animals, Chiang Mai Zoo, Thailand according to procedures described by Brown et al. (2005). In brief, the plasma  $E_2$  EIA utilized a polyclonal antibody ( $E_2$ -R4972, 1:50,000 dilution) which cross-reacted with  $E_2$  at 100% and estrone at 3.3% with  $0.005 \text{ ng}\cdot\text{mL}^{-1}$  low.

#### **Ovary histological examination**

A histological examination was conducted at the veterinary clinical diagnostic laboratory, Khon Kaen University. Ovaries were fixed with a 10% formalin solution for 24 h, dehydrated in ethanol series, cleared in methyl salicylate prior to infiltration, and embedded in paraffin according to a protocol described by Burke et al. (1972). Longitudinal sections of  $4 \text{ }\mu\text{m}$  thickness were stained with hematoxylin and eosin. Ovarian developmental stages were classified as described by Degani and Boker (1992).

##### **Primary growth phase**

Stage I: Chromatin nucleolar stage ( $55\text{-}95 \text{ }\mu\text{m}$  diameter), a large nucleus in a central position with a relatively small ooplasm.

Stage II: Peri-nucleolar stage ( $130\text{-}160 \text{ }\mu\text{m}$  diameter), a larger area of ooplasm in the nuclear.

##### **Secondary growth phase or vitellogenic phase**

Stage III: Cortical alveoli stage ( $220\text{-}325 \text{ }\mu\text{m}$ ), a present of cortical alveoli at the periphery of the oocyte.

Stage IV: Vitellogenesis ( $590\text{-}715 \text{ }\mu\text{m}$ ), oocyte filled with cortical alveoli and deposited yolk granules.

##### **Final oocyte maturation (FOM) phase**

Stage V: Maturation stage ( $640\text{-}745 \text{ }\mu\text{m}$ ), migration of nucleus (Germinal vesicle; GV) to a periphery of the oocyte and dissolution of the nucleus membrane (Germinal vesicle breakdown; GVBD). The deposition of yolk granules increases supplanting the cortical alveoli and ooplasm to the periphery of the oocyte.

Stage VI: Ripe egg stage ( $690\text{-}765 \text{ }\mu\text{m}$ ), a completed displacement of the cortical alveoli and ooplasm to the thin peripheral zone of the oocyte by the yolk granules.

Stage VII: Ripe egg stage. In the process of ovulation.

Oocyte diameter was measured under a microscope (Eclipse Ci-L with DS-U3, Nikon, Japan) by DS-Fi2 software on digital images. Five random positions were taken from each slide for ovarian developmental classification and a calculation for oocyte percentage.

#### **Statistics**

Data are presented as mean $\pm$ SE values. Differences between groups were determined by independent samples t-test, with a statistical significance level at  $p\leq 0.05$ .

## **RESULTS AND DISCUSSION**

**Induced off-season maturation by a long-photoperiod combined with a high water temperature**

The experiment was carried out from January to March which is a winter to early summer season in Thailand. So it is actually three months before a normal spawning season for snakeskin gourami which is from June to September (Tuncharoen et al. 2018). Thus, the experiment was considered as an off-season spawning. The photoperiod and water temperature records are presented in Figure 1. Fish in the ALP+AT group was exposed to the long-photoperiod and high water temperature as 18L:6D, 28.5±0.1°C, while the NC group was exposed to the natural photoperiod and ambient water temperature as 11.5L: 12.5D, 24.8±0.3°C. In the last two weeks of the experiment, there was a slight surge of water temperature from 23.2±0.2°C to 26.4±3.1°C recorded in the NC group (Figure 1b) which was a result of a rise of ambient temperature.

We demonstrated that the female snakeskin gourami exposed to ALP+AT for a 60 days experimental period developed to a full maturation earlier than that of the controlled group (NC group). Plasma E<sub>2</sub>, GSI value, and FOM percentage obtained from the ALP+AT group were significantly higher than that of the NC group after exposure to the long-photoperiod and high water temperature ( $p \leq 0.05$ ) (Figure 2).

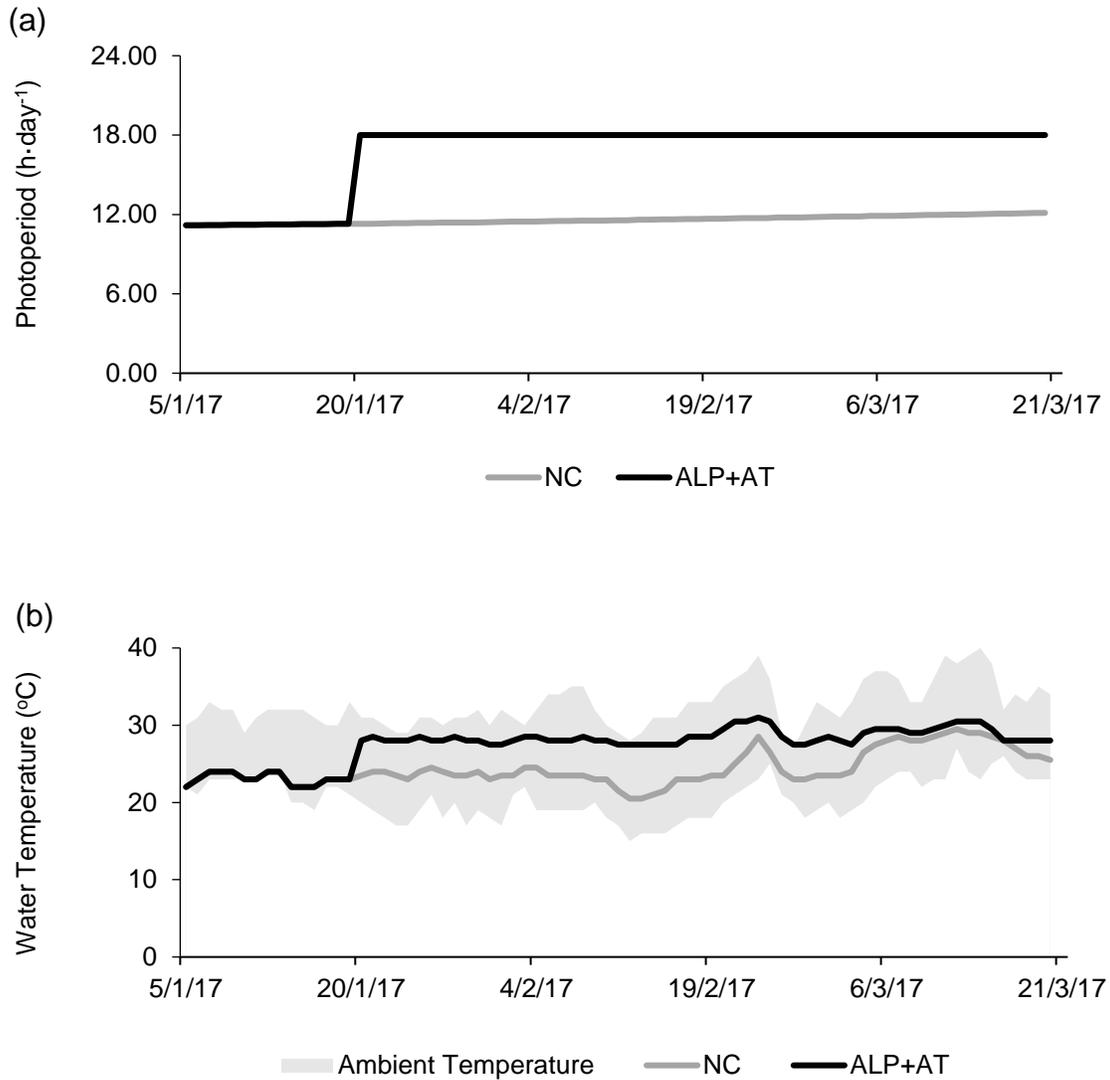
The results from our study are consistent with the previous reports on tropical freshwater fish include *C. catla*, *L. rohita*, *C. mrigala* (Sarkar et al. 2010), *C. auratus* (Sarkar and Upadhyay, 2011), and *C. batrachus* (Acharia et al. 2014). It is generally known that a mechanism of action is regulated through the neuroendocrine system in the fish brain and gonadal development, respectively. The long-photoperiod and high water temperature directly inhibit arylalkylamine N-acetyltransferase (AANAT) activity in the pineal organ leading to an inhibition of the melatonin (N-acetyl-5-methoxytryptamine) production (Maitra et al. 2006; Falcon et al. 2007; 2010). Melatonin is an important hormone that inhibits gonadal development via the dopaminergic system in the hypothalamus. Since melatonin is inhibited, the gonadotropin-releasing hormone (GnRH) is conversely released from the pituitary gland to activate gonadotropin hormone (GtH) production leading to gonadal development (Popek et al. 2006; Falcon et al. 2007, 2010; Migaud et al. 2010; Zohar et al. 2010). Since melatonin is released at

night, exposure to long-photoperiod and high water temperature inhibits melatonin production which in turn allows a secretion of GtH from the pituitary gland triggering oogenesis. During vitellogenesis, the ovaries were triggered by GtH leading to E<sub>2</sub> synthesis leading to a vitellogenin production as yolk precursor (Bobe and Labbé, 2010; Lubzens et al. 2010; Mylonas et al. 2010; Urbatzka et al. 2011). Therefore, elevated levels of plasma E<sub>2</sub> is important evidence suggesting gonadal development as a result of a long photoperiod and a high water temperature in our study.

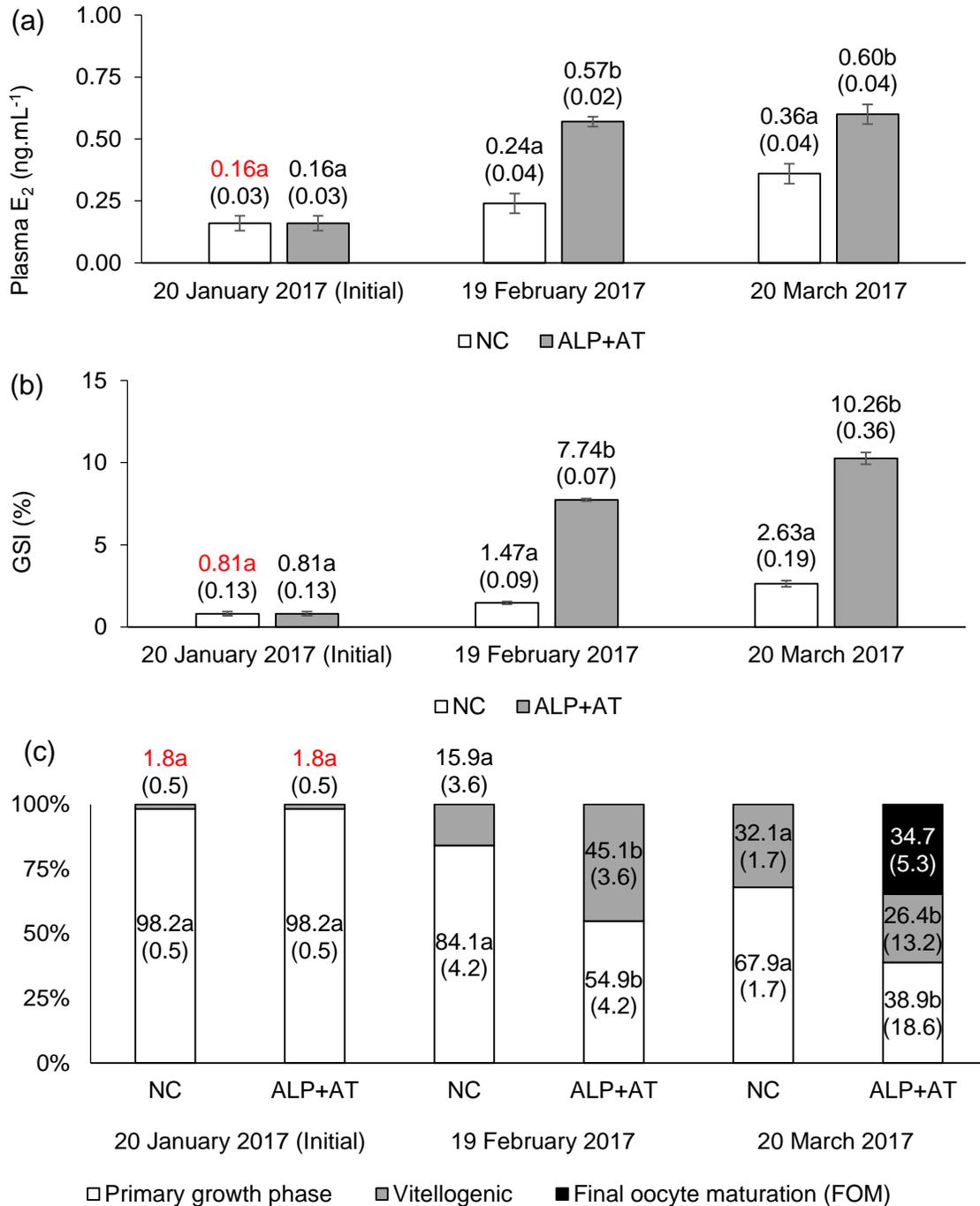
Plasma E<sub>2</sub> obtained from ALP+AT group was drastically increased from 0.16±0.03 ng·mL<sup>-1</sup> at the initial of the experiment to 0.57±0.02 ng·mL<sup>-1</sup> and 0.60±0.04 ng·mL<sup>-1</sup> in day 30<sup>th</sup> and day 60<sup>th</sup>, respectively. It was significantly higher than that of the NC in both day 30<sup>th</sup> and day 60<sup>th</sup> ( $p \leq 0.05$ ), while a gradual increase was observed in the NC group (Figure 2a). The rise of plasma E<sub>2</sub> is in agreement with its role in vitellogenesis (Bobe and Labbé, 2010; Lubzens et al. 2010; Mylonas et al. 2010; Urbatzka et al. 2011).

As a consequent of high plasma E<sub>2</sub> in the ALP+AT group, ovarian development of this group approach to a vitellogenic stage earlier than the controlled NC group. As a result, GSI value in the ALP+AT group was dramatically increased from 0.81±0.13% at the initial of the experiment to 7.74±0.07% and 10.26±0.36% in day 30<sup>th</sup> and 60<sup>th</sup>, respectively. In addition, GSI value in both day 30<sup>th</sup> and 60<sup>th</sup> are significantly higher than its controlled ( $p \leq 0.05$ ) (Figure 2b). Furthermore, the highest GSI value (10.26±0.36%) obtained from day 60<sup>th</sup> in ALP+AT group is almost the same with the peak GSI value obtained from the riverine mature female snakeskin gourami during their natural spawning season (June to October, GSI 11.9±1.8% and 10.9±2.1%) reported by Tuncharoen et al. (2018) and Amornsakun et al. (2004). The result of the histological examination of day 60<sup>th</sup> sample showed that the FOM was observed only in the ALP+AT group at 34.7±5.3%, but not from the NC group where vitellogenic phase was observed at 32.1±1.7% (Figure 2c). These also confirm a role of elevated plasma E<sub>2</sub>.

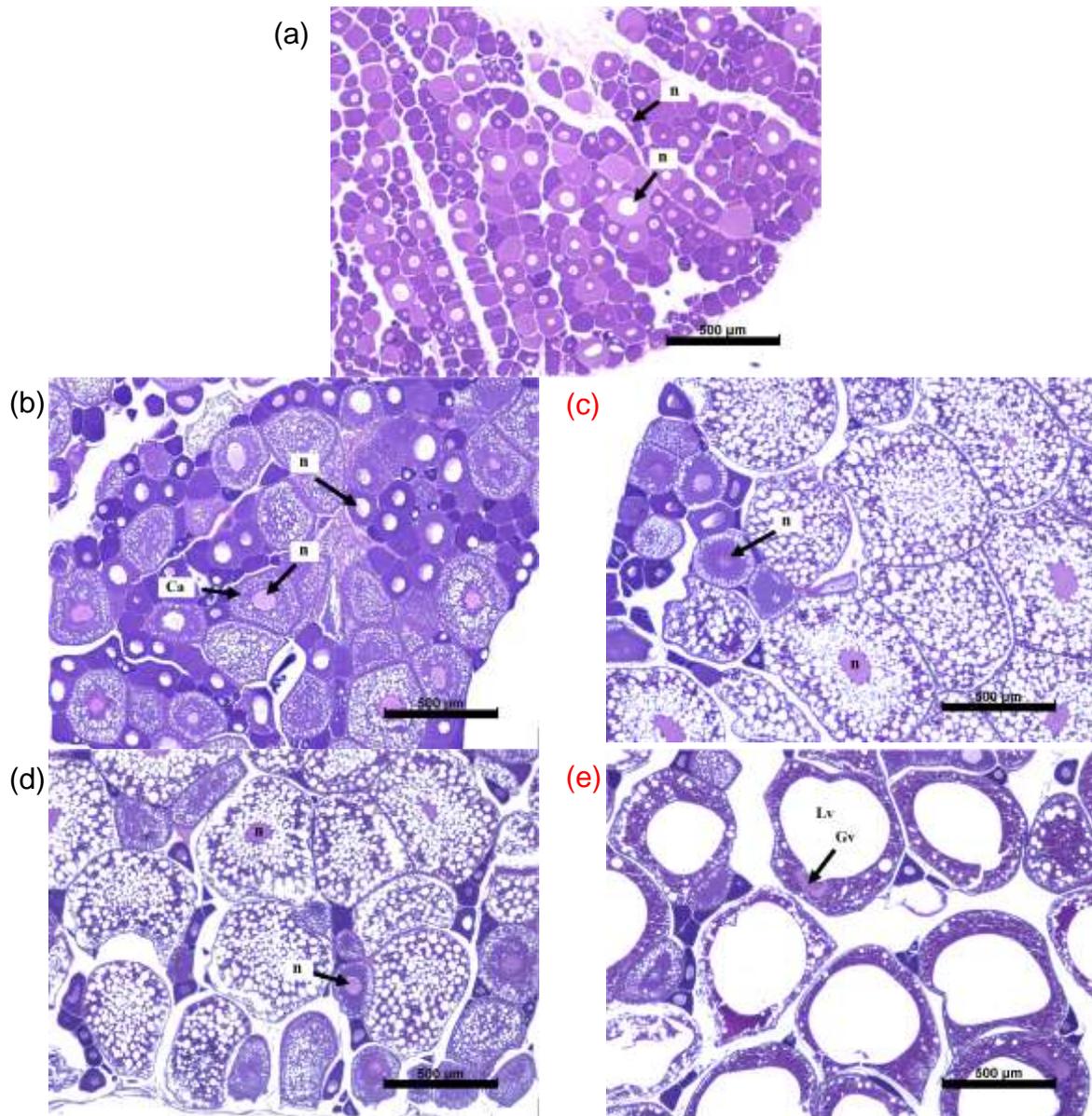
Although gonadal development of the NC group is in slow progress compared to the ALP+AT, it appears that gonadal development of the NC group gradually develops throughout a 60 days experimental period based on parameters shown in Fig 2A, 2B and 2C.



**Figure 1: Illustration of recorded photoperiod and water temperature: (A) Photoperiod and (B) water temperature (5 January to 20 March 2017) of natural condition (NC; 11.5L:12.5D, 24.8±0.3°C) and artificial long-photoperiod combined with artificial water temperature (ALP+AT; 18L:6D, 28.4±0.1°C).**



**Figure 2: A comparison of selected female reproductive indices: (A) Plasma 17β-Estradiol (E<sub>2</sub>) level, (B) gonado-somatic index (GSI) and (C) percentage of various ovarian development phases of female snakeskin gourami (*T. pectoralis*) exposed to natural condition (NC; 11.5L:12.5D, 24.8±0.3°C) and artificial long-photoperiod combined with artificial water temperature (ALP; 18L:6D, 28.4±0.1°C). The means values (SE) is presented above the box. Different letters behind the means indicate significant different (t-test p≤0.05).**



**Figure 3: Representative of ovaries histology sections obtained from snakeskin gourami (*T. pectoralis*) exposed to natural condition (NC; 11.5L:12.5D, 24.8±0.3°C) and artificial long-photoperiod combined with artificial water temperature (ALP; 18L:6D, 28.4±0.1°C). H&E stain; n= nucleolus, Ca= cortical alveoli, Gv= germinal vesicle, Lv= lipid vesicle. Scale bar = 500 µm. (A) Initial ovaries at primary growth phase on 20 January 2017, (B and C) vitellogenic ovaries of NC and APL+AT group respectively on 19 February 2017 after 30 days experiment and (D and E) ovaries in vitellogenic phase of NC and final oocyte maturation (FOM) phase of APL+AT group respectively on 20 March 2017 after 60 days experiment (before breeding).**

Cause of that changes is not clear, however we anticipate that a rise in ambient and a water temperature recorded in the last two weeks of the experiment partly had contributed to that development. Combined with the ALT+AT result, this confirms that the gonadal maturation of

snakeskin gourami can be induced with a long-photoperiod and a high water temperature manipulation.

Base on ovarian morphological changes of both treated groups shown in Figure 3, it was found that the snakeskin gourami has an asynchronous

ovary type, the so-called a multiple spawner. Various ovarian developmental stages were observed throughout the ovary. There is no significant difference ( $p>0.05$ ) in a developmental percentage obtained from five random positions of each slide. Histological analysis of initial ovary on 20 January 2017 contains  $98.2\pm 0.5\%$  of the primary growth phase oocytes, shown in Figure 3a. On 30<sup>th</sup> day of the NC and ALP+AT groups (Figure 3b and 3c), while the NC group begin to develop into the cortical alveoli stage (Stage III), a complete vitellogenic oocytes (Stage IV) is present in the ALP+AT group. Later on the 60<sup>th</sup> day (Figure 3d and 3e), the ovaries of the NC group show  $32.1\pm 1.7\%$  of the cortical alveoli oocyte (Stage III) developed to vitellogenic oocytes (Stage IV). On a contrary, the  $34.7\pm 5.3\%$  of FOM (stage VI) in the ALP+AT group was observed which distinguished by a present of a large lipid vesicle (Lv) at the center of the oocyte, a germinal vesicle (Gv) migrated to the periphery, and a cortical alveoli was displaced by yolk granules. These suggest that a long-photoperiod combined with a high water temperature manipulation for 60 days is sufficient to accelerate gonadal development to the FOM which is ready for a further ovulation.

#### Induced off-season spawning using a synthetic hormone

The result of the hormone-induced off-season spawning is present in Table 1. All six female from the ALP+AT group ovulated within 48 h after hormone injection. Conversely, there was no evidence of ovulation in the NC group. Five out of six pairs of the ALP+AT group that ovulated eggs was successfully fertilized with an average egg production at  $2,318\pm 262$  egg-spawner<sup>-1</sup>, with  $62.0\pm 16.9\%$  hatching rate. These results confirm that snakeskin gourami treated by ALP+AT provides a full maturation during an off-season spawning. On the other hand, the female of the NC group fail on ovulate. A cause of unfertilized from one out of six pairs from the ALP+AT group is not known. There is a possibility of a dominant aggressive males on a territorial defense behavior lead to a reduced sexual behavior (Degani and Ziv, 2016).

We demonstrated that a long-photoperiod combined with a high water temperature manipulation for 60 days for snakeskin gourami resulting in a full gonadal maturation, successful ovulation and fertilization similar to a few species reviewed above (Dey et al. 2004; 2005; Biswas et al. 2005; El-Sayed and Kawanna, 2007; Sarkar et al. 2010; and Sarkar and Upadhyay, 2011).

Although fecundity and fertilization rate in our study is relatively lower than that suggested by Amornsakun et al. (2004), this possibly dues to a smaller broodstock and an off-season spawning condition.

Therefore, and the off-season spawning is an additional snakeskin gourami seed production to a conventional production contributing to marginal productivity.

**Table 1: A comparison of size and selected breeding response parameters of snakeskin gourami (*T. pectoralis*) natural condition (NC; 11.5L:12.5D,  $24.8\pm 0.3^\circ\text{C}$ ) and artificial long-photoperiod combined with artificial water temperature (ALP; 18L:6D,  $28.4\pm 0.1^\circ\text{C}$ ).**

Parameters	Treatment	
	NC	ALP+AT
Sample size	n = 6	n = 6
Male		
Total length (cm)	$11.45\pm 0.30^a$	$11.82\pm 0.22^a$
Body weight (g)	$25.84\pm 0.85^a$	$26.70\pm 0.61^a$
Female		
Total length (cm)	$13.73\pm 0.33^a$	$14.01\pm 0.13^a$
Body weight (g)	$38.01\pm 0.86^a$	$38.89\pm 0.87^a$
Spawn success (pair)	0	6
Fertilization success (pair)	0	5
Egg production (egg-spawner <sup>-1</sup> )	0	$2,318\pm 262$
Hatch rate (%)	0	$62.0\pm 16.9$
Fertility (hatched embryos-spawner <sup>-1</sup> )	0	$1,442\pm 338$

**Note:** Mean $\pm$ SE with the same letters in the row indicate no significant different (t-test  $p>0.05$ ).

#### CONCLUSION

This experiment clearly demonstrated that the reproductive development of snakeskin gourami can be manipulated by a long-photoperiod and a high water temperature. The artificial long-photoperiod (18L:6D) combined with high water temperature ( $28^\circ\text{C}$ ) manipulation for 60 days accelerated gonadal development of snakeskin gourami to maturation, ovulation, and fertilization, respectively. Further study with more sample size to reconfirm if the manipulation used in this study is an effective approach for the snakeskin gourami during an off-season spawning.

#### CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

#### ACKNOWLEDGEMENT

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#### AUTHOR CONTRIBUTIONS

TR designed, performed the experiments and prepared and reviewed the manuscript. TA, SA, and KS carried out sample collection. RP designed experiment, prepared and reviewed the manuscript. All authors read and approved the final version.

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