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Effects of luteinizing hormone-releasing hormone analogue (LHRHa) on ovulation and spawning of *Schistura kohchangensis*

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Abstract It was found that females injected with distilled water did not spawn, while concentrations of LHRHa with DOM clearly showed effectiveness and reliability as an inducing agent for successful ovulation and spawning in *S. kohchangensis*. One hundred percent ovulation was achieved from females receiving all treatment levels of LHRHa plus DOM. A dose of 20 μg·kg^{-1} LHRHa with 10 mg·kg^{-1} DOM gave the shortest latency period and highest relative fecundity. There were no significant differences in percentage of spawning success, fertilization, hatching and relative fecundity among groups of fish injected with 10, 15 and 20 μg·kg^{-1} LHRHa ($p>0.05$). There was also no effect on embryonic development among the three different dosage groups with LHRHa. The study results suggest that 20 μg·kg^{-1} LHRHa plus 10 mg·kg^{-1} DOM is proven to be an efficient and reliable inducing agent for successful ovulation and spawning in *S. kohchangensis*.

Keywords: Induce spawning, LHRHa, Ovulation, *Schistura kohchangensis*

Introduction

Schistura kohchangensis (Smith, 1933), known as Koh Chang Island loach or Ornate tiger sand loach, was first discovered on Koh Chang Island, Thailand. It is a freshwater fish in the loach family Balitoridae, native to rivers in Chanthaburi, Rayong, and Trat provinces of Thailand, and the northern end of the Cardamon Mountain range of south-western Cambodia (Kottelat, 1990). The Koh Chang Island loach is a small, slender, bottom-dwelling fish, with an elongated body. The body has a pale white to golden background with 10–12 dark vertical bars (Kottelat, 1990; Rainboth, 1996). Its habitat is normally shallow, often high-gradient streams with moderate to fast–flowing waters, containing substrates of gravel, rocks and boulders. Its natural diet consists of insect larvae and algae (Kottelat, 1990; Nithirojpakdee *et al*., 2012).

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Although *S. kohchangensis* is not an economically important fish for consumption, it is popular as an ornamental aquarium fish (Panitvong, 2020). Wild populations of *S. kohchangensis* are being greatly reduced, due to overfishing for the ornamental fish trade, by impacts from environmental change, such as streams drying up during the arid season, and by the expansion of farmland. In order to conserve this species in natural waters, breeding studies are required, which will assist to increase productivity of this species. Presently there are no reports that describe the breeding season of *S. kohchangensis*. Our results, from monitoring gonadosomatic index (G.S.I index) throughout a year from 234 *S. kohchangensis*, found that G.S.I of male fish peaked in May and was lowest in October. Whereas, the G.S.I of female fish was highest in June and also lowest in October. This indicates that this fish species tends to reproduce and spawn only once a year. Our findings are consistent with Termwichakorn and Suksri (2011) who reported that breeding season of fish in the Balitoridae family occurs during rainy season (May to August). There are no known reports describing techniques for breeding this particular species of loach. This study is aimed to determine the best protocol to increase numbers of *S. kohchangensis*, raised from a hatchery setting, then released back into the original broodstock water source. This study will not only serve to help protect a local aquatic animal from extinction and preserve biodiversity, but also provide technical guidance to those interested in producing this fish commercially for the ornamental trade.

When wild fish are released into a hatchery setting as broodstock, they often exhibit some form reproductive dysfunction, such as failure to naturally spawn. Dysfunction may be due to the fish being in captivity and not experiencing the conditions and cues of the spawning grounds. The pituitary gland can also fail to release maturational gonadotropin or luteinizing hormone (Peter, 1983). Environmental and hormonal manipulation have been utilized to stimulate reproductive processes, by inducing gonadal maturity and by stimulating ovulation or spermiation and improve spawning under hatchery conditions (Zohar and Mylonas, 2001; Asturiano *et al.*, 2005; Bosak-Kahkesh *et al.*, 2010). In Thailand, induction of ovulation by luteinizing hormone-releasing hormone analogue (LHRHa) is a common technique, used to manage reproduction of commercial fish (Zohar, 1996; Na-Nakorn, 1987). It has been used successfully to induce spawning in many fish species, including dwarf loach, *Yasuhikotakia sidthimunki* (Phrarom *et al*., 2003); red-tailed mystus, *Hemibagrus wyckiodes* (Singsee and Udomkarn, 2004); snail eater, *Pangasius conchophilus* (Sahatnarepaipong *et al*., 2004); stonelapping minnow, *Garra cambodgiensis* (Pornsopin and Joradol, 2006); stone loach, *Schistura poculi* (Pornsopin *et al*., 2015) and mini dragon loach, *Schistura pridii* (Pornsopin *et al*., 2020). However, there are no reports of aquarium spawning nor published studies

on the effects of LHRHa on breeding and spawning of Koh Chang Island loach. Therefore, the objective was to investigate the effects of different dosages of LHRHa on spawning performance in wild-caught *S. kohchangensis*.

Materials and methods

Experimental design

Effects of LHRHa on spawning performance of *Schistura kohchangensis* were studied using a completely randomized design (CRD). Four concentrations of LHRHa (0, 10, 15 and 20 $\mu g \cdot kg^{-1}$) were tested, with three replications (one female *S. kohchangensis* as a replicate). The experiment was performed at the Rajamangala University of Technology Tawan-ok Chanthaburi Campus, Thailand.

Experimental fish

Adult wild-caught *S. kohchangensis* were collected from Klong Takhian canal, Chanthaburi Province, Thailand, during pre-spawning season (January-April, 2016). Two hundred fish of unknown age were obtained, with average total lengths of approximately 3.5-4.0 cm. These were transported to the Rajamangala University of Technology Tawan-ok, Chanthaburi Campus and initially stocked in 120 cm Ø circular concrete tanks with mixed sexes, at a density of 100 fish per square meter. Fish were fed ad libitum twice a day with frozen bloodworms (larvae of *Chironomus* sp.). All broodstock tanks were maintained in an indoor hatchery. Water was continuously aerated and temperature was maintained at 26±1°C. The tanks were cleaned by siphoning every three days, with approximately 50% of the 200-liter water volume exchanged. Fish were acclimated in this tank system for eight months before being used in the induced breeding experiment.

After eight months, mature broodfish were selected for use in the induced breeding experiment. At sexual maturity, mature males could only be identified by emission of sperm with gentle pressure to the abdomen. Ripe females were selected according to the criteria of a well-rounded and soft belly, erect genital papilla, and visible small white oocytes in the abdomen (Figure 1b) (Pornsopin *et al*., 2015; Pornsopin *et al*., 2020).

Breeding tank preparation

Twelve glass aquaria (60×30×36 cm) were used for breeding. All aquaria were cleaned and dried two days before use. Aeration was provided by a pump,

into all tanks, using one air stone (ø 5 cm) placed in the center of each aquarium. Clean freshwater was added to all tanks, through a filter net, ensuring a volume of 43 L for each tank.

Experimental procedure

Twelve pairs of mature female and male *S. kohchangensis* with average body weights of 1.80±0.30 g and 1.78±0.28 g, respectively, were induced to spawn using single injections of LHRHa (Suprefact®) and domperidone (Motilium-M®, Janssen-Cilag, Thailand). Fish in the control group were injected with distilled water only. Injections were administered intramuscular with the assigned hormone dosage as shown in Table 1. Prior to handling, each fish was first anesthetized in a clove oil bath (30 $mg \cdot L^{-1}$).

After hormonal injection, subject fish were stocked in a 20×20×20 cm plastic net, hung inside the breeding tanks, using a one breeding pair per net. There, they were allowed to spawn naturally. After spawning, the broodfish were removed from each net, and spawned eggs were incubated at an ambient temperature of 26±1ºC. The number of spawned fish, latency period, interval between injection and spawning, and number of spawned eggs were recorded. A hundred spawned eggs were sampled from each breeding aquaria and transferred to a 10 L clear glass jars, containing 5 L water, supplied with aeration through sand stones. These holding jars provided direct observation of simultaneous hatching, for estimating fertilization, hatching and survival rates. Thirty fertilized eggs were sampled from each treatment and stocked in petri dishes (n=360). They were monitored under the stereomicroscope starting from the 4-cell stage every 15 min until hatching. Each embryonic development stage was photographed, and duration was recorded. Percentage of fertilization was determined by hand counting under the microscope, after fertilized eggs had developed into the gastrula phase. Fertilization success (%) was determined for each treatment from three sub-samples of eggs (n = 100), by dividing the number of fertilized eggs by the number of eggs sampled. The number of eggs and hatched larvae were hand counted in each tank. Hatching success (%) was calculated as the number of hatched eggs, per total number of fertilized eggs. Spawning success (%) was calculated as the spawned fish per total treatment fish. Larval survival was recorded after the fry reached three days old, and survival rate was calculated, using the number of 3-days old larvae per total number of hatched eggs.

Statistical analysis

Treatments were compared in terms of latency period, number of spawned fish, relative fecundity, fertilization rate, hatching rate and survival. The means were compared by analyzing the variance of arcsine transformation of the percentage data. Significant differences in means between pairs of treatments were evaluated with Duncan's new multiple Range Test. All comparisons were considered to be significant when $p<0.05$.

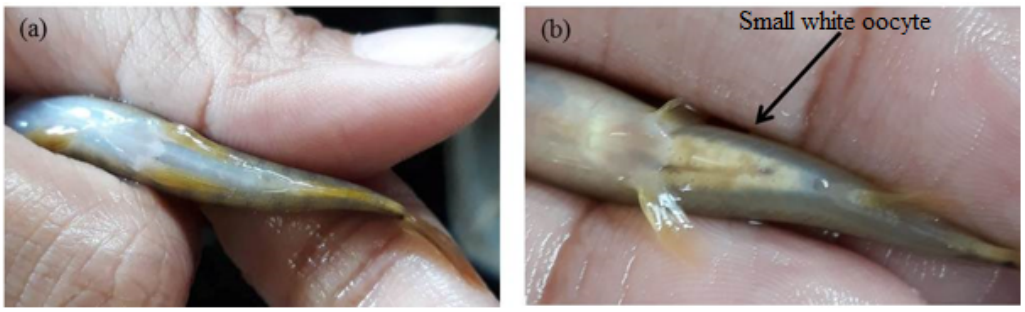

Figure 1. Ventral surface of mature *S. kohchangensis* broodfish: (a) male; (b) female

Table 1. Dosage of LHRHa (Suprefact®) used for induced spawning of *Schistura kohchangensis*

Treatment	**Hormonal dosage**	
	Female	Male
T_1 (control)	Distilled water	Distilled water
T_2	LHRHa at 10 μg·kg^{-1} + domperidone at 10 mg·kg^{-1}	LHRHa at 10 μg·kg^{-1} + domperidone at 10 mg·kg^{-1}
T_3	LHRHa at 15 μg·kg^{-1} + domperidone at 10 mg·kg^{-1}	LHRHa at 10 μg·kg^{-1} + domperidone at 10 mg·kg^{-1}
T_4	LHRHa at 20 μg·kg^{-1} + domperidone at 10 mg·kg^{-1}	LHRHa at 10 μg·kg^{-1} + domperidone at 10 mg·kg^{-1}

Results

Spawn was obtained approximately 7-9 hours after females were injected with LHRHa in all experimental groups. None of the females injected with distilled water (control) spawned. The average spawning success was 100±0.00 percent across all experimental groups. Treatment with LHRHa at 20 μg·kg^{-1} resulted in the shortest latency period of 7.17±0.20 hours ($p\leq0.05$), compared to treatments of 15 μg·kg^{-1} (9.10±0.17 h) and 10 μg·kg^{-1} (8.41±0.08 h). The relative fecundity in 15 μg·kg^{-1} LHRHa treated female was not significantly different from that of 10 or 20 μg·kg^{-1} LHRHa treated group ($p>0.05$), although relative

fecundity in the 20 μg·kg^{-1} LHRHa treated group was significantly higher than that of 10 μg·kg^{-1} LHRHa treated group (p≤0.05) (Figure 2).

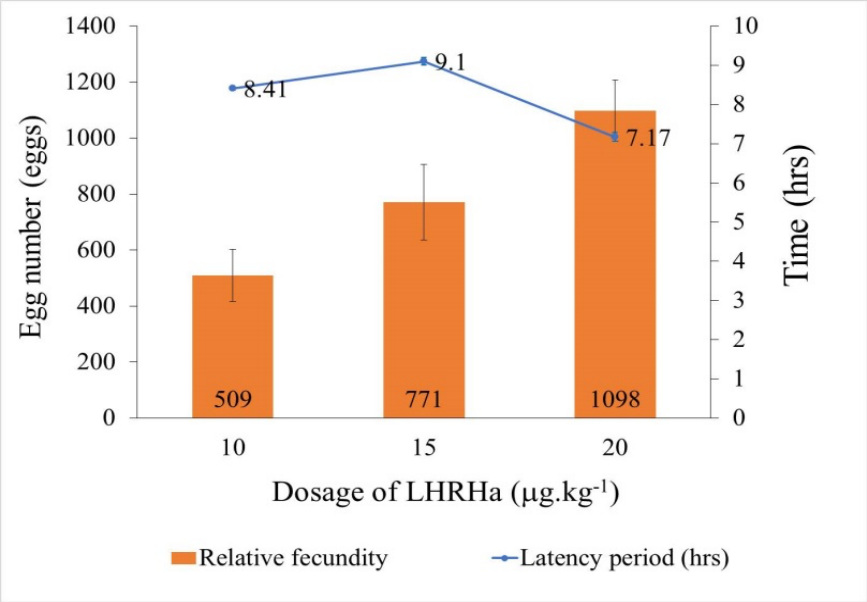

Figure 2. Latency period and relative fecundity of brood females injected with 10, 15 and 20 μg·kg^{-1} LHRHa: Bars and line represent mean±SE; Significant differences in the means are indicated by different letters above the error bars ($p<0.05$; $n = 3$)

There were no significant differences in fertilization, hatching and larvae survival among treated fish injected with 10, 15 and 20 μg·kg^{-1} LHRHa ($p>0.05$) (Figure 3). The fertilized eggs of *S. kohchangensis* were round in shape, with yellow-orange color, demersal and non-adhesive, with diameters ranging from 1.49 to 1.51 mm. Development of the embryo after fertilization is summarized as follows: the cleavage period was completed at 1 h and 20 min post fertilization. Morula stage ended at 1 h and 25 min, blastula stage at 2 h and 36 min, and then gastrula stage at 4 h and 13 min. Somite development started (12- somite) at 9 h and 18 min and the 22-somite stage were completed at 10 h 56 min. Hatching took almost 18 h at 25-26 °C water temperature. The early-stage larvae generally swam to the bottom of the aquaria.

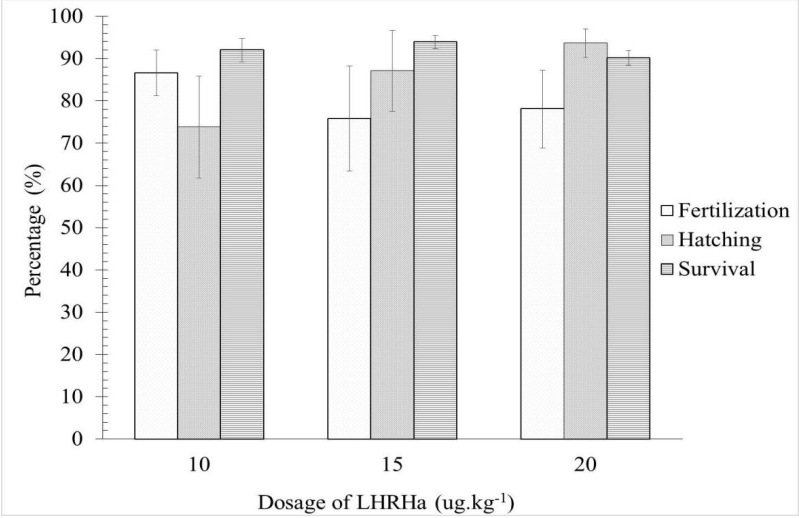

Figure 3. Fertilization, hatching and survival (mean±SE) of female *S. kohchangensis* injected with 10, 15 and 20 μg·kg^{-1} LHRHa

Discussion

After vitellogenesis, oocyte maturation, ovulation and spawning of fish reared in captivity can be induced by hormone treatment (Bhattacharya *et al*., 2018). However, appropriate hormone type and concentration needs to determined, to reach oocyte maturation in vivo and efficient reproduction periods. In this study, LHRHa (Suprefact®) plus domperidone (Motilium-M®) proved to be effective in inducing ovulation of the Koh Chang Island loach, *S. kohchangensis*, and resulted in the production of spawned eggs of an overall high quality. Percentage of spawned females was high (100%), with desirable rate of fertilization, hatching and larvae survival (range from 76-87%, 74-94% and 90-94%, respectively). These parameters would indicate successful spawning inducement, since it reflects the completion of oocyte maturation. Our results show much better spawning success than Pornsopin *et al*. (2015), who reported ovulation of 25-75% in *S. poculi*, for females treated with LHRHa and domperidone. This difference may be due to the levels of LHRHa used, since our study tested this hormone at 10-20 μg·kg^{-1}, while Pornsopin *et al*. (2015) used 2.5-10 μg·kg^{-1}. They found spawning percentage for *S. poculi* was highest (75%) with satisfactory fertilization (81%) and hatching (60%) when 10 μg·kg^{-1} LHRHa was used. Only 25%-37.5% spawning was achieved in females treated with 2.5-5.0 μg·kg^{-1} LHRHa, without any fertilization or hatching. Insufficient exposure to hormone levels likely resulted in an inability to stimulate release of eggs from the follicle (Na-Nakorn, 1995), and therefore ovulation did not occur completely.

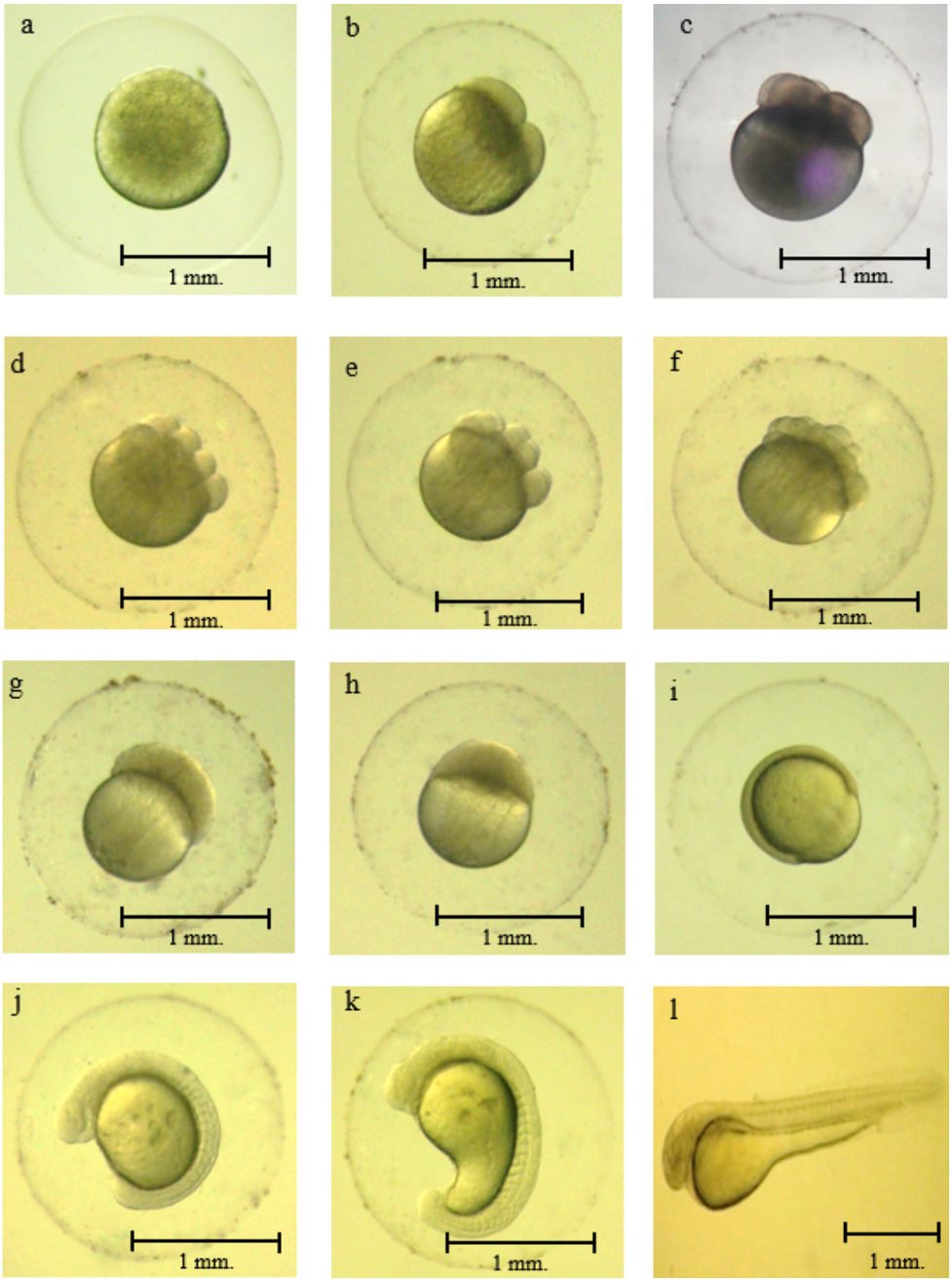

Figure 4. Embryonic development stages of fertilized eggs of Koh Chang Island loach, *S. kohchangensis* (Smith, 1933): (a) Fertilized egg; (b) 2–cell; (c) 4–cell; (d) 8–cell; (e) 16–cell; (f) 32–cell; (g) morula; (h) blastula; (i) gastrula; (j) somite; (k) heart formation; (l) hatch-out

The success of induced breeding is sometime dependant on latency period (Rahdari *et al*., 2014). The latency period, time between the first hormonal injection and ovulation, is often related to water temperature, with the period decreasing with an increase in temperature (Kupren *et al*., 2011). In this study we investigated latency time at same temperature, with different hormone concentrations. The latency period varies greatly among fish species; therefore, knowing the exactly latency period is very helpful for induced breeding by hormone injection and naturally spawning methods. This knowledge allows operators to immediately separate the broodstock from the breeding tank before they damage the fertilized eggs. Finding from this study showed that a concentration of 20 $\mu g \cdot kg^{-1}$ LHRHa gave the shortest latency period and highest relative fecundity. This may due to levels of 20 $\mu g \cdot kg^{-1}$ LHRHa, provided higher secretion of gonadotropin in the blood of *S. kohchangensis*, strongly inducing most oocyte in the ovarian follicle to become mature, further synchronizing ovulation before eventually spawn. Therefore, this study suggests single injections of LHRHa and domperidone at 20 $\mu g \cdot kg^{-1}$ and 10 $mg \cdot kg^{-1}$, respectively, proved to be an efficient and reliable inducing agent for successful ovulation and spawning in *S. kohchangensis*. This is in contrast to many other species in the Cypriniformes order that respond to LHRHa and domperidone at lower dosages, such as *Gyrinocheilus aymonieri* (Apithanakun, 1997), *Garra parvifilum* (Phrarom *et al*., 2002) and *Yasuhikotakia sidthimunki* (Phrarom *et al.*, 2003). LHRHa can be retained in the blood for 30 minutes-15 hr (Barannikova *et al*.,1982) or 10-23 minutes (Gothif and Zohar, 1991). It can stimulate fish to secrete gonadotropins and has effects of stimulating development of oocytes and ovulation and semen secretion (Peter, 1983). *S. kohchangensis* is a small-bodied fish; therefore, a single-dose hormone injection for ovulation helps decrease handing stress and trauma. This has been noted for other a small-bodied fish, such as *G. aymonieri* and *G. parvifilum*, where spawning success was achieved using a single injection (Apithanakun, 1997; Phrarom *et al*., 2002).

S. kohchangensis is relatively easy to breed. After induced spawning using LHRHa, these fish ovulate and spawn within 7-9 hours. Their spawning response after hormone injection is similar to tiger loach, *Botia helodes* (Promprasert *et al*., 2011) and red-tail sand loach, *S. mahnerti* (Kantiyawong *et al*., 2017). However, spawning of *S. kohchangensis* occurred sooner than in other loaches of the same genus, such as *S. poculi* (Pornsopin *et al*., 2015) and *S. pridii* (Pornsopin *et al*., 2020).

Eggs of *S. kohchangensis* are round in shape with yellow-orange color, demersal and non-adhesive, similar to *S. pridii* (Pornsopin *et al*., 2020) and *S. poculi* (Pornsopin *et al*., 2015). Hatching occurs approximately 17 hours after fertilization at a water temperature of 26±1 ^{o}C, similar to *S. mahnerti,* (18 hours

at 26.33±0.94 ºC). *S. kohchangensis* has a faster hatching time than *S. poculi* (Pornsopin *et al*., 2015) and *S. pridii* (Pornsopin *et al*., 2020). This is most likely due to water temperature differences in their respective habitats. A comparison of reproductive performance among loaches, using LHRHa and DOM for induced spawning, is presented in Table 3. Length of embryonic development stages and time of hatching were similar to those of other loaches. This similarity in development can be attributed to a close evolutionary relationship among species.

Table 3. Comparison of reproductive performance among female loaches, when induced to spawn using LHRHa ($ug \cdot kg^{-1}$) in combination with domperidone ($mg \cdot kg^{-1}$)

Information	*S. poculi* (Pornsopin *et al*., 2015)	*S. mahnerti* (Kantiyawong *et al*., 2017)	*S. pridii* (Pornsopin *et al*., 2020)	*S. kohchangensis* (this study)
Hormone Dose	10/10	10/10	10/5	20/10
Temperature (ºC)	17-18	26.33±0.94	15.3-16.8	26±1
No. of eggs	105±28	657.5±115.59	149.75±22.75	1,098±191.25
Spawning time (h)	10-12	6	12-13	7.17±0.2
Hatching time (h)	44	18	80	18
Fertilization rate (%)	81.09±4.99	74.5±10.11	35.25±28.48	78.11±15.96
Hatching rate (%)	59.96±3.95	67.16±19.9	31.75±27.26	93.63±5.79
Survival rate (%)	47.36±7.36	96.0±3.40	86.25±7.09	90.14±2.98

Hormone dosage of LHRHa did not affect ovulation, fertilization rate, hatching or embryonic development of *S. kohchangensis*, at the levels tested. Therefore, this study concludes that induced spawning using single injections of 20 $\mu g \cdot kg^{-1}$ LHRHa and 10 $mg \cdot kg^{-1}$ DOM is the optimal strategy for inducing natural spawning in mature wild-caught Koh Chang Island loach, *S. kohchangensis*.

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