Breeding Performance and Embryonic Development of *Bithynia siamensis goniomphalos*, the First Intermediate Host of *Opisthorchis viverrini*

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ABSTRACT

The freshwater snail, *Bithynia siamensis giomphalos*, is the first intermediate host of liver fluke (*Opisthorchis viverrini*) in northeast Thailand. Wild broodstock of the snails from three dams, namely Nong Harn Dam (NH), Lampao Dam (LA), and Ubonrat Dam (UB) were collected and identified by sex. The male and female snails (2:3 sex ratio) were mated in a glass box (10x5x5.5 cm) in three replicates. The average number of spawned eggs per day was highly significant in NH population (6.48-13.90 eggs) ($P<0.05$) compared with LA (4.57-9.22 eggs) and UB (2.70-5.63 eggs) populations. The hatching rate of *B. s. goniomphalos* larva for 30 days was 92.18, 90.94, and 93.07 % in LA, NH, and UB populations, respectively. The hatching and survival rates of *B. s. goniomphalos* larva were similar among the three populations ($P>0.05$). The embryonic development composed of 5 stages, namely cleavage, morula, trochophora, veliger, and hippo stages. The breeding performance and embryonic development of snails may be essential for the control of *O. viverrini* prevalence.

Keywords: Breeding performance, embryonic development, *Bithynia siamensis goniomphalos*, liver fluke

INTRODUCTION

Opisthorchiasis, together with associated cholangiocarcinoma, is one of the human parasitic diseases occurring in the lower Mekong area, e.g. Thailand, Lao PDR, Cambodia, and Vietnam (Waikagul, 1998; Sripa *et al*., 2011; Sitthithaworn *et al*., 2012). The liver fluke, *Opisthorchis viverrini*, can infect and inhabit within the intrahepatic bile ducts of humans after eating undercooked fish (Grundy-Warr *et al*., 2012). The eggs of liver fluke released from mammal feces can transform into sporocyst and cercariae. *Bithynia siamensis goniomphalos*, a freshwater snail, is recognized as the specific host of transmission in the life cycle of the liver fluke (*Opisthorchis viverrini*) (Petney *et al*., 2012).

The initial transmission of *O. viverrini* is with the first intermediate host, *B. s. goniomphalos*. Increased population of the specific host of *O. vivierrini* can accelerate *O. viverrini* prevalence. The more the first intermediate host (snail) is present in the

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environment, the higher the distribution of liver flukes. This phenomenon was supported by Kiatsopit *et al*. (2012) who explained that the highest number of *B. s. goniomphalos* was found in Sakon Nakorn province, and the infection of *O. viverrini* only occurred in the Songkram wetland (Sakon Nakorn and Nakhon Phanom provinces), while no infection occurred in Buriram, Surin, Chaiyaphum, Kalasin, Mukdahan, and Mahasarakham wetlands. Similarly, Sripa *et al* (2011) also reported that Sakon Nakorn had the highest incidence of cholangiocarcinoma (CCA). Moreover, the Bureau of Epidemiology (2006) reported that Sakon Nakorn had the highest rate of incidence of opisthorchiasis in 2003 and 2005.

The genus *Bithynia*, a gastropod mollusk belonging to the family Bithyniidae, is the first intermediate host of liver fluke (*Opisthorchis viverrini*). The three species of this genus, namely *B. funiculata*, *B. s. siamensis*, and *B. s. goniomphalos*, are distributed in various areas in Thailand (Petney *et al*., 2012; Kiatsopit *et al*., 2013). *B. s. goniomphalos* was found in Northeast part of Thailand, i.e. Khon Kaen, Kalasin and Sakon Nakorn provinces (Brandt, 1974; Brokelman *et al*., 1986; Chitramvong, 1992; Suwannatrai *et al*., 2011) which were associated with the prevalence of liver fluke infection (Sithithaworn *et al*., 2012). *B. s. gonionphalos* can be found on muddy artificial and natural ponds, swamps, as well as on vegetation (Lohachit, 2004-2005). Lohachit (2004-2005) carried out habitat studies on *B. s. goiomphalos* in Khon Kaen province between June 1989 and May 1990, and reported that this species was present in a wide range of environmental conditions. They were found at water temperatures from 18 to 33^{o}C, turbidities from 8 to 450 FTU, pH from 6.3 to 8.5, and dissolved oxygen from 2 to 10 ppm. *B. s. goniomphalos* was found at more than 1.5 m depth, while *B. funiculata* was present at water depths of 30 cm (Ngern-klun *et al*., 2006) and no more than 45 cm (Chitramvong, 1991).

Bithynia snails are oviparous animals which can spawn eggs (3-8 eggs per set) on natural materials (rocks and gravels). At 27-28^{o}C, the development period of *B. s. goniomphalos* eggs ranged from 10 to 12 days (Kruatrachue *et al*., 1982). There are some breeding works of this species represented in studies of Kaewpan (1982) and Brockelman *et al*. (1986). *B. s. goniomphalos* production in laboratory can serve as animal model and fulfill sustainable control of opisthorchiasis. Limited information is available about breeding ability and embryonic development of snails.

The aims of this study were to provide clarification and obtain a better understanding of the reproductive performance, spawning rate, hatching period, and mortality rate of *B. s. goniomphalos* embryo in each population, and to monitor embryonic development of snail eggs at room temperature.

MATERIALS AND METHODS

Bithynia samples

One hundred *B. s. goniomphalos* samples were collected from three natural waters, namely, Lampao Dam (LA, Kalasin province), Nong Harn Dam (NH, Sakon

Nakorn province), and Ubonrat Dam (UB, Khon Kaen province) which were infested with liver fluke in July 2013 (Figure 1). The snail samples were then transported to the Fisheries Laboratory, Faculty of Agriculture, Ubon Ratchathani University, where they were acclimatized in aquarium (24x28x30 cm). The identification of sex was carried out under light in the laboratory (Chitramvong, 1992). The mono-sex snail was raised in the glass aquarium and fed with artificial feed modified from Kaewpan (1982) until the female released the batch of eggs fertilized from unknown males in the natural waters.

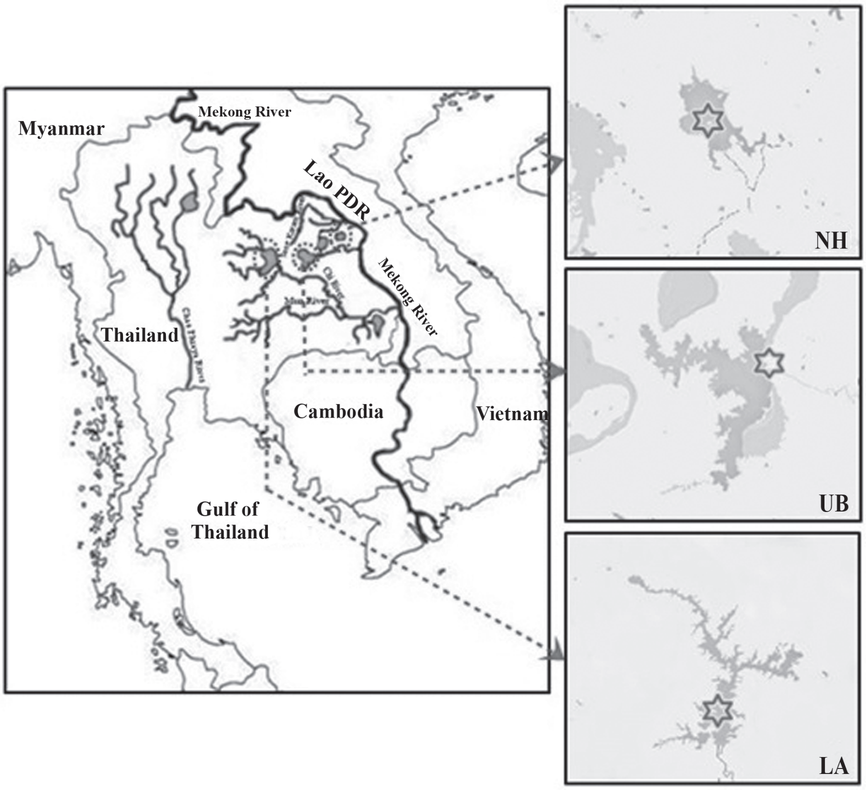

Figure 1. The location of sampling sites for *B. s. goniomphalos* in three reservoirs in Northeast of Thailand. Codes for sampling sites are as follows: Nong Harn Dam (NH), Lampao Dam (LA), and Ubonrat Dam (UB).

Feed, soil, and water preparation

Feed was prepared by mixing all the ingredients listed in Table 1, then heated until boiling. The mixture was stored at 4^{o}C. Leaf lettuce was chopped into tiny pieces, soaked in KMnO4, boiled for 3-4 min, and ground with 800 mL of cold water. The 22 mL of mixture and 16.5 mL of leaf lettuce solution were mixed, afterwhich 250 mL of distilled water was added.

Table 1. Composition of raw materials for feed

Components	Weight/Volume
Rice bran	21.0 g
Leucaena leaf meal	21.0 g
Casein	10.0 g
Calcium carbonate	0.7 g
Vitamin mix	2.0 g
Agar	0.7 g
Distilled water	500.0 mL

Loamy sand was prepared for spawning and hatching studies of *B. s. goniomphalos*. The dry loamy sand was ground, after which calcium carbonate (1 kg of loamy sand : 20 g of calcium carbonate) was added. The loamy sand powder was autoclaved at 120^{o}C for 3 hours and placed at room temperature. The water in all experiments was mixed with the following chemical ingredients; ferric chloride 0.25 g, calcium chloride 11 g, magnesium sulfate 10 g, phosphate buffer 1.25 mL (potassium acid phosphate 34 g, 1N sodium hydroxide until pH was 7.2), and distilled water to 1 L.

Breeding studies

The breeding experiment was composed of three treatments (LA, NH, and UB populations) with 4 replications. The tank bottom was spread with the prepared loamy sand at 0.5 cm depth, and water depth was maintained at 5 cm during the breeding period. The randomly selected male and female *B. s. goniomphalos* (0.103-0.215 and 0.131-0.263 g body weight, respectively) were collected and transferred to a plastic box (10x5x5.5 cm) for acclimatization. The male and female were mated at the ratio of 2:3. The number of spawned eggs per day was monitored at 28±1^{o}C for 60 days after fertilization.

Triplicate aquaria were utilized for nursing the three larval groups. One hundred fertilized eggs of the three *B. s. goniomphalos* populations were divided into aquaria and recorded as percent of hatching and survival rate at 28±1^{o}C for 30 days.

Embryonic development studies

The fertilized eggs of *B. s. goniomphalos* were pooled and transferred into an aquarium in the laboratory with controlled temperature (28±1^{o}C). The stages of embryonic development were recorded and photographed under the microscope until the eggs hatched.

Data analysis

Data are presented as means and standard deviations of *B. s. goniomphalos* broodstock. Box plots were drawn and medians of total number of spawned eggs per day, hatching rate, and survival rate were calculated for differences among three populations using the R software environment. Significant differences in body weight of broodstock and breeding performances (total spawned eggs per day, hatching rate, and survival rate) among the three populations were determined using analysis of variance (ANOVA) at confidence level of 95 % using the R software environment (R Development Core Team R, 2006).

RESULTS

Breeding performance

B. s. goniomphalos obtained from the wild could survive and accept artificial feed in the experimental aquarium. After fertilization, the eggs were spawned on the 7th day in NH population, while the eggs were found on the 8th day in LA and UB populations. The females in all populations could spawn until 60 days. The total number of spawned eggs per female reached 419-834 in NH population, while the spawned eggs per female ranged from 274-553 and 162-338 eggs in LA and UB populations. The maximum number of eggs per female reached 153, 130, and 81 eggs in LA, NH, and UB populations, respectively (Figure 2). The number of spawned eggs per day were highly significant in NH population (6.48-13.90 eggs) ($P<0.05$) when compared with LA (4.57-9.22 eggs) and UB (2.70-5.63 eggs) populations.

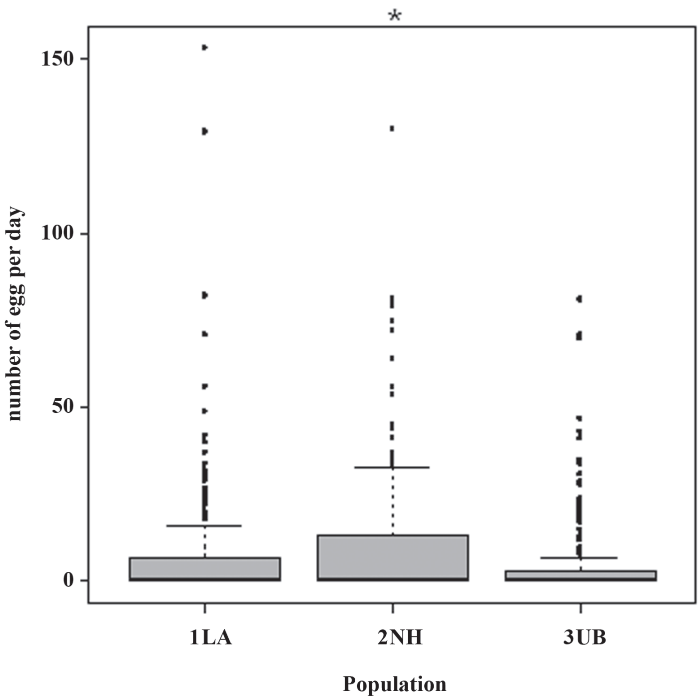

Figure 2. The spawned eggs per day of *B. s. goniomphalos* in laboratory at 28±1^{o}C for 60 days. Codes for populations are as follows: Nong Harn (NH), Lampao (LA), and Ubonrat (UB) reservoirs. The asterisk indicates significant difference (P<0.05).

Hatching occurred on the 7th day in NH and UB populations, while it was on the 8th day in LA population. The hatching period was similar (6-7 days) in all populations. Hatching rate per day ranged from 1.03-46.36, 0.33-39.90, and 0.48-32.92 in LA, NH, and UB populations, respectively (Figure 3). The average hatching rates were 92.18, 90.94, and 93.07 % in LA, NH, and UB populations, respectively. No significant difference in hatching rates was found among the populations (P>0.05). The survival rate of *B. s. goniomphalos* larva was also similar among the three populations (P>0.05) (Table 2).

Table 2. Survival rate of *B. s. goniomphalos* larva for 30 days in laboratory at 28±1^{o}C.

Population	Survival rate (%)
Lam Pao Dam (LA)	68.8±3.3
Nong Harn Dam (NH)	63.5±3.9
Ubonrat Dam (UB)	67.97±2.9

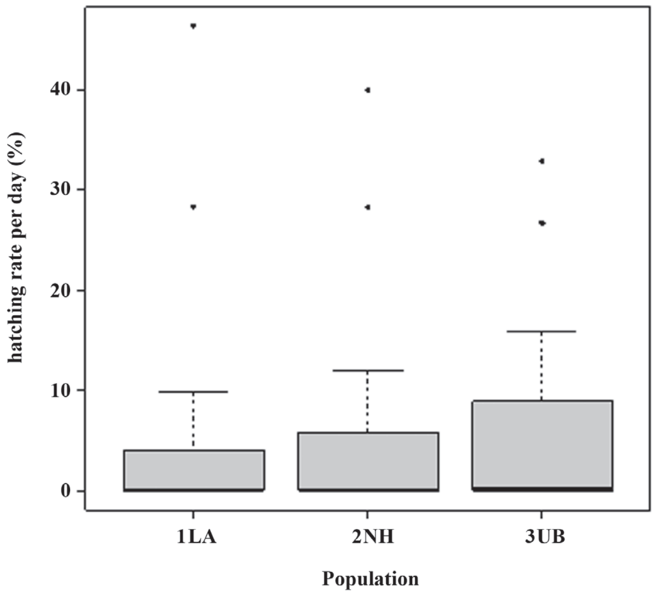

Figure 3. The hatching rate per day of *B. s. goniomphalos* eggs in laboratory at 28±1^{o}C. Codes for populations are as follows: Nong Harn (NH), Lampao (LA), and Ubonrat (UB).

Embryonic development

The embryonic development of *Bithynia* was divided into five stages, namely, cleavage, morula, gastrula, trochophore, veliger, and hippo stages. Three to four rows of fertilized eggs were laid and attached tightly on the bottom of the aquarium (Figures 4a and 4b). The yellowish zygote was encapsulated within gel-like substance to protect the eggs (Figures 4c and 4d). Due to the transparent gel cover, the embryonic development was clearly observed under the microscope. The cleavage stage began 2 hours after spawning. At the end of the first cleavage stage, the two blastomeres almost separated. At 12 hours, the morula stage showed a blastocoel. At 26 hours, the trochophore stage showed a whitish and grainy embryo. The veliger stage lost the round form and eyes appeared at 54 hours. At 102 hours, the embryo developed into the hippo stage completely for the formation of eyes and heart (Figure 5).

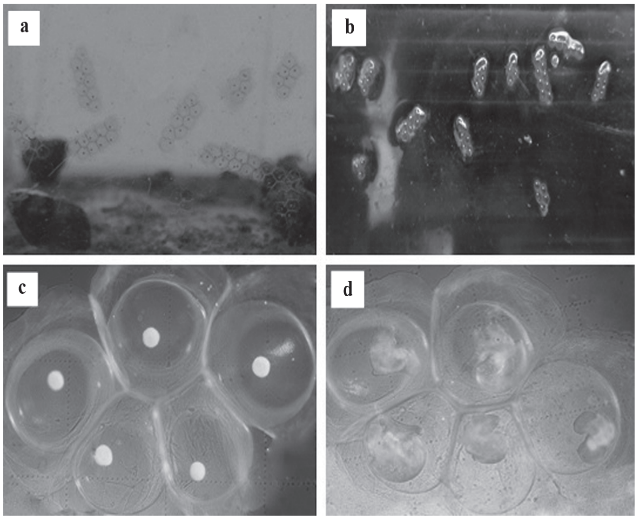

Figure 4. *B. s. goniomphalos* eggs at the bottom of the aquarium

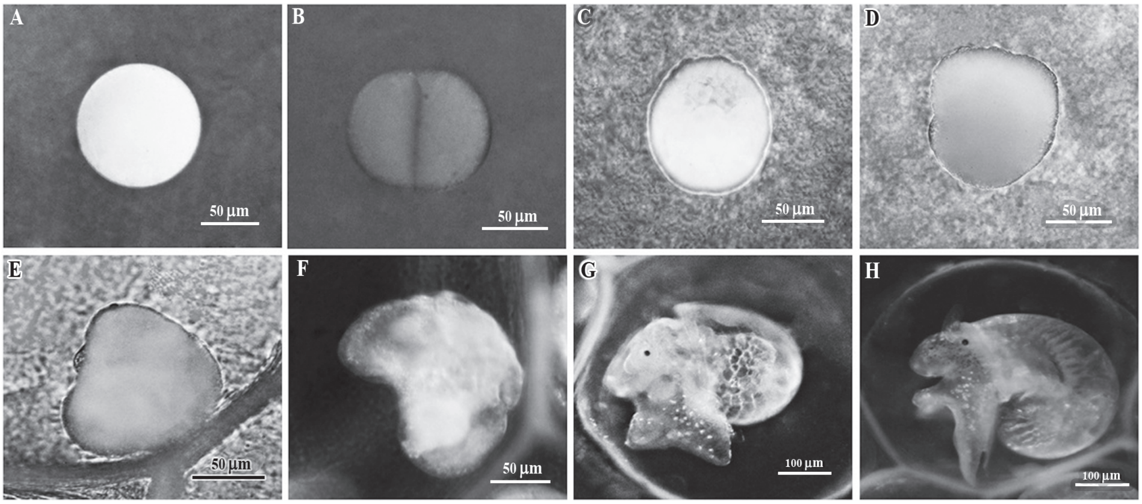

Figure 5. The embryonic development of *B. s. goniomphalos* as observed under the microscope. A=zygote (1 hr); B=cleavage stage (3 hrs); C=morula stage (12 hrs); D=trochophora stage (32 hrs); E=early veliger stage (63 hrs); F=late veliger stage (72 hrs); G=early hippo stage (117 hrs); H=late hippo stage (182 hrs).

DISCUSSION

The characterization of pond bottom in Ubonrat Dam was muddy, while the benthic zone in Nong Harn and Lampao Dams was sandy and silty. Light could penetrate through until the pond bottom. Normally, *B. s. goniomphalos* are found abundantly in ponds, rice fields, and streams in Northeast Thailand (Kaewpitoon *et al*., 2008; Suwannatrai *et al*., 2011; Kiatsopit *et al*., 2012), while Wang *et al*. (2015) explained that *B. s. goniomphalos* were highly abundant in rice paddy fields. The increase in *Bithynia* populations may be specific to the benthic condition in natural ponds because they prefer slow flowing water environments and muddier and finer substrates (Petney *et al*., 2012). Forbes and Lopez (1990) studied the habitat of deposit-feeding gastropod (*Hydrobia truncata*) and reported that sandy habitats can stimulate better growth more than coarse-grained sediments.

The significant number of spawned eggs was found in NH population. The differences in spawned eggs per day might be due to the body weight of female broodstock associated with fecundity (Table 3). Snails in NH wetland had the greatest breeding performance and they preferred the artificial environment in aquarium. Dillen *et al*. (2010) reported that the body weight of land snail (*Succinea putris*) broodstock affected fecundity and spawning in the laboratory, while the body size of freshwater snail (*Helisoma trivolvis*) influenced the total number of eggs and number of eggs per egg mass (Norton and Bronson, 2006).

Table 3. Body weight of *B. s. goniomphalos* broodstock

Station	Body weight (g)	
	Female	Male
Lam Pao Dam (LA)	0.156 ± 0.026^{b}	0.147 ± 0.016^{ab}
Nong Harn Dam (NH)	0.216 ± 0.037^{a}	0.175 ± 0.037^{a}
Ubonrat Dam (UB)	0.168 ± 0.037^{b}	0.138 ± 0.020^{b}

Different superscript letters indicate that body weights of the three broodstock populations were significantly different ($P<0.05$).

The breeding performance of *B. s. goniomphalos* may depend on geological habitat of population. The major wetlands in northeast Thailand belong to two basins, namely, Sakhon Nakorn basin which was the habitat of NH population, and Korat basin which was the habitat of LA and UB populations (Suwannatrai *et al*., 2011). Additionally, Kiatsopit *et al*. (2013) studied the population variation of *B. s. goniomphalos* in Thailand and Lao PDR using allozyme markers, and reported that snails could be divided into two groups, composing of populations from Songkram and Nam Ngum wetlands (NH population) and Chi and Mun wetlands (LA and UB populations).

The total number of spawned eggs of *B. s. goniomphalos* per female in this study (LA, NH, and UB=274-553, 389-834, and

162-338 eggs, respectively) were lower than what Singkachart (1989) previously reported, which was 1,457 eggs per female, at 27-28^{o}C.

The hatching time of *B. s. goniomphalos* in this study (NH and UB=7th-13th; LA=8th -13th) was faster than what was reported by Brockelman *et al*. (1986). Moreover, the survival rate of *B. s. goniomphalos* larva for 30 days (LA, NH, and UB= 68.8±3.3, 63.5 ±3.9 and 67.97±2.9 %, respectively) was highly significant when compared with the study of Brockelman *et al*. (1986) for 120 days (28.3 %). These differences may be due to the genetic information of *B. s. goniomphalos*, water and feed management, and environmental conditions in the laboratory.

The development period and characterization of *B. s. goniomphalos* embryo depends on species and environmental conditions, especially temperature. The characteristic features represented in this study are similar to those of *Lymnaea stagnalis*, having five stages of embryonic development: cleavage, morula, trochophora, veliger, and hippo stages, and egg capsules attaching tightly to the substrate (Gomot, 1998). The period of embryonic development in the three populations ranged from 7 to 13 days, while that of *Lymnaea stagnalis* took place over a period of 10 to 20 days (Lalah *et al*., 2007; Bandow and Weltje, 2012).

B. s. goniomphalos acst as the first intermediate host of *O. viverrini* and is distributed over the lower Mekong basin. According to the findings in this study, NH broodstock presented the highest body weight which reflected greater breeding performance. Kiatsopit *et al*. (2012) reported that the number of *O. viverrini* cercariae released is positively associated with the size of *B. s. goniomphalos*. Furthermore, they reported that the highest percent of snails infected with *O. viverrini* cercariae was found in Sakon Nakorn province (104-551 snails), including those in Nong Harn wetland. The biological varieties of the snail population are critical component for more knowledge in regulating *O. viverrini* prevalence.

ACKNOWLEDGEMENT

We sincerely thank Dr. Pradon Chatikavanij who supported the idea and information involving parasitic studies. We also thank the Faculty of Agriculture, Ubon Ratchathani University.

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