Correlation of Spawning Season and Maturational Parameters, Expression Levels of Vitellogenin Genes in *Notopterus notopterus* and *Anematichthys armatus* in Kwan Phayao, Thailand

Dutrudi Panprommin[1*,2], Santiwat Pithakpol[1], Siriluck Valunpion[1] and Kanyanat Soontornprasit[1]

ABSTRACT

The partial cDNA sequences of the vitellogenin (VTG) gene were cloned from the liver tissues of *Notopterus notopterus* and *Anematichthys armatus*. The expression levels of VTG could not be directly used for investigation of the spawning season of the two fish species, but we can indirectly rely on their expressions as the information indicates relation with spawning season. The gonadosomatic index (GSI) and hepatosomatic index (HSI) were also examined for analysis of the seasonal changes in the two fish species. Fish samples were collected from June 2012 to May 2013. Significant variations of VTG expression levels ($P<0.05$) were observed during the spawning season, from January ($0.42 \pm 0.07\%$) to August ($0.97 \pm 0.15\%$), and April ($0.20 \pm 0.09\%$) to September ($1.04 \pm 0.14\%$) for *N. notopterus* and *A. armatus*, respectively. The relationships between VTG genes expression, GSI and HSI values were determined using correlation analysis. The VTG expression levels have a significant ($P<0.01$) direct correlation with the mean GSI values. The highest mean GSI values were also observed during the spawning season: August and September for *N. notopterus* and *A. armatus*, respectively. The mean HSI value has an inverse trend to the VTG expression and the mean GSI value. Thus, the VTG expression profile and the GSI values reflect the seasonal spawning on an annual basis of two fish species in Kwan Phayao.

Keywords: spawning season, vitellogenin, *Notopterus notopterus*, *Anematichthys armatus*, Kwan Phayao

INTRODUCTION

Kwan Phayao is the largest lake in northern Thailand. It is located in Muang district, Phayao province and covers an area of 2.3 km^2. The northern part of the lake is fed by the Ing river and 12 surrounding streams. The area around Kwan Phayao is utilized for various purposes. The western areas of the lake are used for rice production

[1] Fisheries, School of Agriculture and Natural Resources, University of Phayao, Phayao 56000, Thailand
[2] Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand
* Corresponding author, email: dutrudeep@yahoo.com

and other agricultural purposes, whereas the eastern side is the town. The water of Kwan Phayao is mainly used as a domestic, raw source for tap water and agriculture. Kwan Phayao is also a major recipient of untreated wastewater from various sources and activities. At present, the water quality of Kwan Phayao is in a polluted condition. Many substances are released into the lake, including heavy metals (Tupwongse *et al*., 2007) and pesticide residues (Sapbamrer and Hongsibsong, 2014). Moreover, microcystins are constantly produced by the algae, *Microcystis* sp., in Kwan Phayao (Whangchai *et al*., 2013). These substances affect aquatic animals, especially their reproductive system (Jones *et al*., 2000; Zhao *et al*., 2014).

Vitellogenins (VTGs) are the major precursor of the egg yolk proteins lipovitellins and phosvitin, which are sources of nutrients during early embryonic development. They are high-density glycolipo-phosphoproteins with a molecular weight of 300-700 kDa (Johnsen *et al*., 1999). VTGs are normally synthesized in the liver tissue of mature females under the control of circulating estrogen, particularly 17β-estradiol (E_2) (Wallace, 1985). E_2 is produced by ovarian follicles in response to the gonadotropin releasing hormone, is secreted into the bloodstream and subsequently travels to the liver tissue. In general, VTG levels tend to increase during vitellogenesis in female fish. The levels of VTG transcript are used as an indicator of the process of oocyte development in fish (Okumura *et al*., 2002; Mikawa *et al*., 2006) and for determination of the spawning season in several fish species, including *Lepisosteus platyrhincus* (Orlando *et al*., 2007) and *Clarias macrocephalus* (Panprommin *et al*., 2008). Moreover, the male and immature female fish can also synthesize VTGs by stimulation with exogenous estrogenic compounds (Okumura *et al*., 2002). Therefore, measuring VTG mRNA expression in the liver is one approach to monitor environmental estrogen exposure (Jones *et al*., 2000; Barucca *et al*., 2006).

In the present study, the VTG expression levels in the liver tissue of females of two fish species, *Notopterus notopterus* and *Anematichthys armatus*, in Kwan Phayao were determined monthly using real-time PCR. This is the first study to investigate partial VTG sequences of these fish and use them for monitoring the annual reproductive cycle. In addition, seasonal changes in the gonadosomatic index (GSI) and hepatosomatic index (HSI) were also determined to analyze their relationship with the VTG mRNA expression.

N. notopterus and *A. armatus* are carnivorous fish in the freshwater food webs. These fish are the predominant species in Kwan Phayao and also commonly found in other rivers. They have commercial importance in the local markets.

Knowing the spawning season of the fish will be useful in managing aquatic animal population for sustainable utilization. Because the fish are reproducing, spawning and larval rearing during this period, the prohibition of fishing affects an increasing number of fish in the water resources.

MATERIALS AND METHODS

Fish samples

Six fish samples of each species were caught monthly from June 2012 to May 2013 from Kwan Phayao, northern Thailand. The average body weights were 166 ± 14.54 and 20 ± 2.60 g in *N. notopterus* and *A. armatus*, respectively. The fish were anesthetized with 0.2 g/l of MS-222 (Sigma, Saint Louis, Missouri, USA) suspension in water and dissected. The whole body of each fish was weighed, then the ovary and liver were removed and weighed for the calculation of the gonadosomatic index [%GSI = (the ovary weight/the body weight) x 100] and the hepatosomatic index [%HSI = (the liver weight/the body weight) x 100] (Htun-Han, 1978). A portion of the liver tissues of the female fish were collected and placed in TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) for extraction of total RNA.

Extraction of total RNA and first strand cDNA synthesis

The liver tissues were first homogenized in 1 ml of TRIzol reagent using glass gliders. The homogenate was incubated at room temperature for 5 min. Two hundred microlitres of chloroform was added and vigorously shaken by hand for 15 sec. Then, the mixture was centrifuged at 12,000 g at 4 °C for 15 min. After the colorless upper phase was transferred to a new 1.5 ml microcentrifuge tube, the total RNA was precipitated by adding 500 µl of isopropanol followed by centrifugation. The quantity and quality of the total RNA was determined by measuring the absorbance at the OD of 260 nm and performing electrophoresis on 1.0% formaldehyde-agarose gel, respectively.

The first-strand cDNAs were synthesized with 1 µg of total RNA from each fish at different time samplings, and 1 µl of oligo (dT_{18}) primer using the iScript™ Select cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The reaction was incubated at 65 °C for 5 min and chilled on ice for 1 min. One microlitre of Moloney murine leukemia virus (MMLV) reverse transcriptase, 4 µl of 5x first-strand buffer and 2 µl of 10 mM dNTPs were added and incubated first at 42 °C for 90 min and then at 85 °C for 5 min. The first strand cDNAs were stored at -20 °C until used.

Cloning of VTG and β-actin partial sequences

The VTG and β-actin sequences of the two fish species have not been reported in the GenBank database. The reverse transcription polymerase chain reaction (RT-PCR) technique was used to investigate these sequences. The VTG sequences were determined using the degenerate primers VF1:VR2 and VF1:VR1 (Barucca *et al*., 2006) for amplification in *N. notopterus* and *A. armatus*, respectively (Table 1). The β-actin sequence of *N. notopterus* was investigated using actin forward and actin reverse primers (Scholz and Gutzeit, 2000) (Table 1). The actin fw2 and actin revC primers (Andreassen *et al*., 2005) were used to amplify the sequence in *A. armatus*.

The RT-PCR reaction mixture in a total volume of 25 µl contained 2.5 µl of

Table 1. Primers used in the amplification of VTG and β-actin for RT-PCR and real-time PCR.

Primer	Sequence for 5' to 3'	Purpose	Reference
VF1	CAGGTNTTRGCWCARGAYTG	Cloning of VTG	Barucca *et al*., 2006
VR1	CCYYTCATCCAGTCNRCAAC	Cloning of VTG	Barucca *et al*., 2006
VR2	AGRMASMACCCAGGARTGVGC	Cloning of VTG	Barucca *et al*., 2006
actin forward	CAGGGAGAAGATGACCCAGAT	Cloning of β-actin	Scholz and Gutzeit, 2000
actin reverse	GATACCGCAGGACTCCATACC	Cloning of β-actin	Scholz and Gutzeit, 2000
actin fw2	TGAAGTACCCCATCGAGCAC	Cloning of β-actin	Andreassen *et al*., 2005
actin revC	CACATCTGCTGGAAGGTGGA	Cloning of β-actin	Andreassen *et al*., 2005
NnVTG-1fq	AGGTTCCTACCACCAGCCTCCCA	real-time PCR	This study
NnVTG-1rq	AGGAGCGAACAGCGACAGACCG	real-time PCR	This study
NnACT-1fq	TCACCACCACAGCCGAGAGGGA	real-time PCR	This study
NnACT-1rq	GAGGAAGCAGCTGTGCCCATTTCC	real-time PCR	This study
AaVTG-1fq	TGGAAGTGCCCATCAGCAGCCT	real-time PCR	This study
AaVTG-1rq	CCTGAAGCCCGTGACTAGGAGCAT	real-time PCR	This study
AaACT-1fq	GGAGATGGGCACCGCTGCTTCT	real-time PCR	This study
AaACT-1rq	GGCCTCTGGGCACCTGAACCTC	real-time PCR	This study

10x *Taq* buffer, 1 µl of 25 mM $MgCl_2$, 1.5 µl of 2.5 mM dNTPs (Fermentas, Vilnius, Lithuania), 1 µl of each 10 µM forward and reverse primer, 0.2 µl of 1 U *Taq* DNA polymerase (Fermentas) and 0.5 µl of first strand cDNA. RT-PCR was performed for 5 min at 94 °C, followed by 30 cycles with 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C. The PCR products were determined by 1.5% agarose gel electrophoresis, stained with ethidium bromide and subjected to UV visualization.

The PCR products were purified using the HiYield™Gel/PCR DNA Fragments Extraction kit (RBC Bioscience Corp., Xindian, New Taipei, Taiwan). Fifteen microlitres of each PCR product were directly sequenced using the Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) at the Macrogen Laboratory (Macrogen Inc., Seoul, Korea). The forward (VF1, actin forward and actin fw2) and reverse (VR2, VR1, actin reverse and actin revC) primers were used for sequencing the 3' and 5' end of the PCR products, respectively.

Sequence analysis

After sequencing, the 3' and 5' sequences were assembled using the Clustal W program (Thompson *et al*., 1994) and subsequently searched the homology using the BLASTn (for nucleotide similarity) and BLASTx (for translated nucleotide similarity) programs (Altschul *et al*., 1990). The VTG partial sequences of *N. notopterus* and *A. armatus* were compared with those of rainbow trout (Mouchel *et al*., 1996) to determine the regions of the domain; heavy chain lipovitellin (LV1), phosvitin (PV) or

light chain lipovitellin (LV2) for confirmation the exact position of primers. To find the positions, the β-actin sequences were compared with the highest similarity sequences in the GenBank database.

The BLASTx program was used for investigating the amino acid position of the cloned VTG sequences of *N. notopterus* and *A. armatus*. The same positions of the amino acid sequences were used to construct the phylogenetic tree with other teleost fish including guppy *Poecilia reticulata* (ABN 80455), swordtail fish *Xiphophorus hellerii* (AFH08752), sheepshead minnow *Cyprinodon variegatus* (AAG30349), Indian carp *Catla catla* (ACN23847), common carp *Cyprinus carpio* (BAD51933), and zebrafish *Danio rerio* (AAW56967) by the neighbor-joining method and bootstrapped for 1000 replicates using MEGA version 4 (Tamura *et al*., 2007). The zebrafish vitellogenin type III (AF254638) was used as an out-group.

Quantification of VTG mRNA by real-time PCR

Quantitative real-time PCR was carried out using the iQ™ SYBR™ Green Supermix (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. One microgram of total RNA was used to synthesize cDNA from each fish sample at different monthly sampling periods and used as a template to determine the relative expression of VTG in females from each fish species. The PCR was performed in a 25 µl volume containing 13 µl of 2x iQ™ SYBR® Green supermix, 1 µl of 5 µM of each primer, and 1 µl of first strand cDNA. The gene-specific primers were designed from nucleotide sequences of mRNA in this study (Table 1). The NnVTG-1fq: NnVTG-1rq and AaVTG-1fq: AaVTG-1rq primers were used for amplification the VTG genes in *N. notopterus* and *A. armatus*, respectively. For β-actin gene, NnACT-1fq: NnACT-1rq and AaACT-1fq: AaACT-1rq primers were used amplified in *N. notopterus* and *A. armatus*, respectively. The level of VTG mRNA expression in each sample was normalized to the expression level of the housekeeping gene β-actin. PCR was performed for 3 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C, 30 sec at 60 °C and 15 sec at 72 °C. After amplification, analysis of the melting curve was continued by holding at 60-95 °C for 10 min. PCR was carried out using a MiniOpticon Real-Time PCR System (Bio-Rad, Hercules, CA, USA). All samples were run in quadruplicate (each duplicate for VTG and β-actin genes).

The threshold cycle (CT) value of VTG and β-actin genes at different time courses was used to calculate the mRNA expression levels. The relative expression of VTG mRNA was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

All data of each fish at different time samplings were presented as means ± standard deviation. Data were analyzed for their variances by one-way analysis of variance (ANOVA), followed by mean comparing with Duncan's multiple range test. Significance was set at $P<0.05$. Correlation analysis was used to test relationships between the GSI values, HSI and the expression levels of VTG gene for each fish species. Pearson

or Spearman rank correlations were tested depending on the distribution of the data. Significance was set at $P<0.01$.

RESULTS

Analysis of VTG and β-actin sequences

The lengths of partial cDNA sequences of VTG and β-actin from the two fish species are shown in Table 2. From the homology search using the BLASTx program, VTG types I and II were amplified in *N. notopterus* and *A. armatus*, respectively. These sequences were determined from regions of the three major domains of VTG gene, heavy chain lipovitellin (LV1), phosvitin (PV) or light chain lipovitellin (LV2). The deduced amino acid sequences of the VTG from the two fish species were compared with rainbow trout VTG (Mouchel *et al*., 1996). These amino acid sequences were placed in the same domain, light chain lipovitellin, at positions 1445-1560 and 1453-1521 in *N. notopterus* and *A. armatus*, respectively.

Table 2. Degenerate primers used for amplification of cDNA sequence, lengths of amplicon fragments and accession number in the GenBank database.

Species	Gene	Primers	Length (bp)	Accession number
N. notopterus	VTG	VF1-VR2	347	KJ626322
	β-actin	actin forward-actin reverse	359	KJ626323
A. armatus	VTG	VF1-VR1	207	KJ626324
	β-actin	actin fw2-actin revC	524	KJ626325

For the β-actin sequences, the deduced amino acid sequences were compared with *Morulius calbasu* β-actin (GenBank accession number AF393832). The positions of the amino acid sequence were 128-275 and 96-295 in *N. notopterus* and *A. armatus*, respectively.

The phylogenetic tree showed that the *N. notopterus* and *A. armatus* VTGs were clustered type I and II groups, respectively. The type I group consisted of guppy *Poecilia reticulata*, swordtail fish *Xiphophorus hellerii* and sheepshead minnow *Cyprinodon variegatus*, whereas Indian carp *Catla catla*, common carp *Cyprinus carpio* and zebrafish *Danio rerio* were grouped in type II (Figure 1). Moreover, these two groups were clearly separated from the zebrafish type III which was used as an out-group.

GSI and HSI in *N. notopterus* and *A. armatus*

The GSI and HSI in *N. notopterus* and *A. armatus* were determined monthly for one year, from June 2012 to May 2013. In *N. notopterus*, the mean GSI values increased significantly ($P<0.05$) from November (1.90 ± 0.31%) to August (9.57 ±0.45%) (Figure 2A). The highest mean GSI value was detected in August. After that, the mean GSI values decreased immediately with the lowest mean GSI value detected in October (0.52 ±0.08%). In contrast, the highest mean HSI value was detected in October (1.81 ± 0.10%), and the

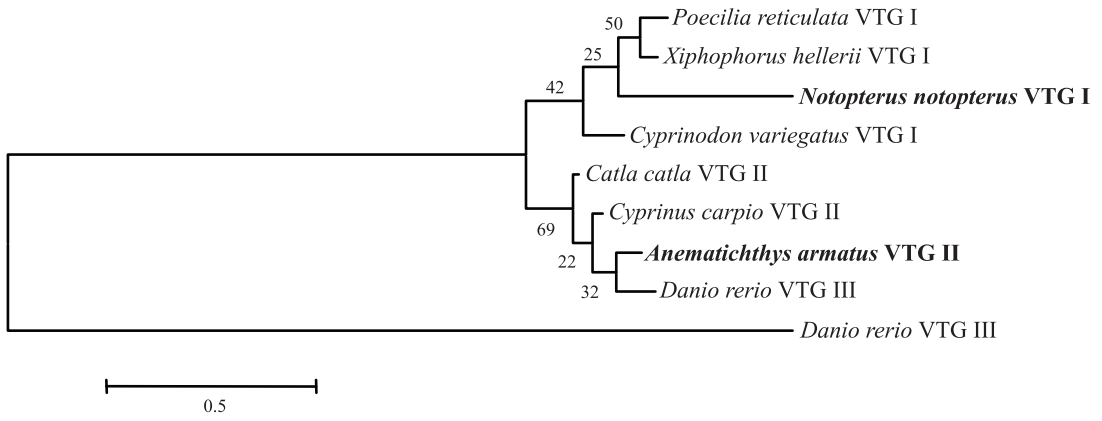

Figure 1. Phylogentic tree of the deduced amino acid sequences of teleost fish VTGs. The numbers on branches indicate bootstrap values after 1000 replications.

lowest mean HSI value (0.66 ± 0.06%) was detected in August (Figure 2B).

For *A. armatus*, the mean GSI values increased significantly (P<0.05) from March (5.84 ± 0.06%) to September (18.58 ± 0.62 %), and the highest value was detected in September (Figure 3A). After spawning, the mean GSI values decreased immediately until the lowest value was detected in November (1.19 ± 0.09%). While the lowest mean HSI value was detected in August (0.68 ± 0.07 %) which was the spawning period, and the highest value was detected in October (1.84 ± 0.07%) (Figure 3B).

Expression of VTG genes in N. notopterus and A. armatus

The relative expression levels of VTG mRNA in females from two fish species were determined monthly using real-time PCR. The relative expression levels of VTG mRNA of *N. notopterus* in the spawning season increased gradually from January (0.42 ± 0.07 %) to August (0.97 ± 0.15%) but increased 48% from July to August (Figure 2C). The highest expression level of VTG was observed in August and decreased 155% from August to September.

For *A. armatus*, the expression levels of VTG increased significantly (P<0.05) from April (0.20 ± 0.09%) to September (1.04 ± 0.14%) (Figure 3C). The highest expression of VTG was detected in September, showing an increase of 34% from August to September, followed by a 333% decrease after spawning from September to October.

Correlation of GSI, HSI and expression levels of VTG genes in *N. notopterus* and *A. armatus*

Based on the distribution of data, Pearson and Spearman correlation analysis were used to test the relationships between the GSI, HSI and expression levels of VTG genes in *N. notopterus* and *A. armatus*, respectively (Table 3). There were significant direct linear correlations between the GSI values and expression levels of VTG gene

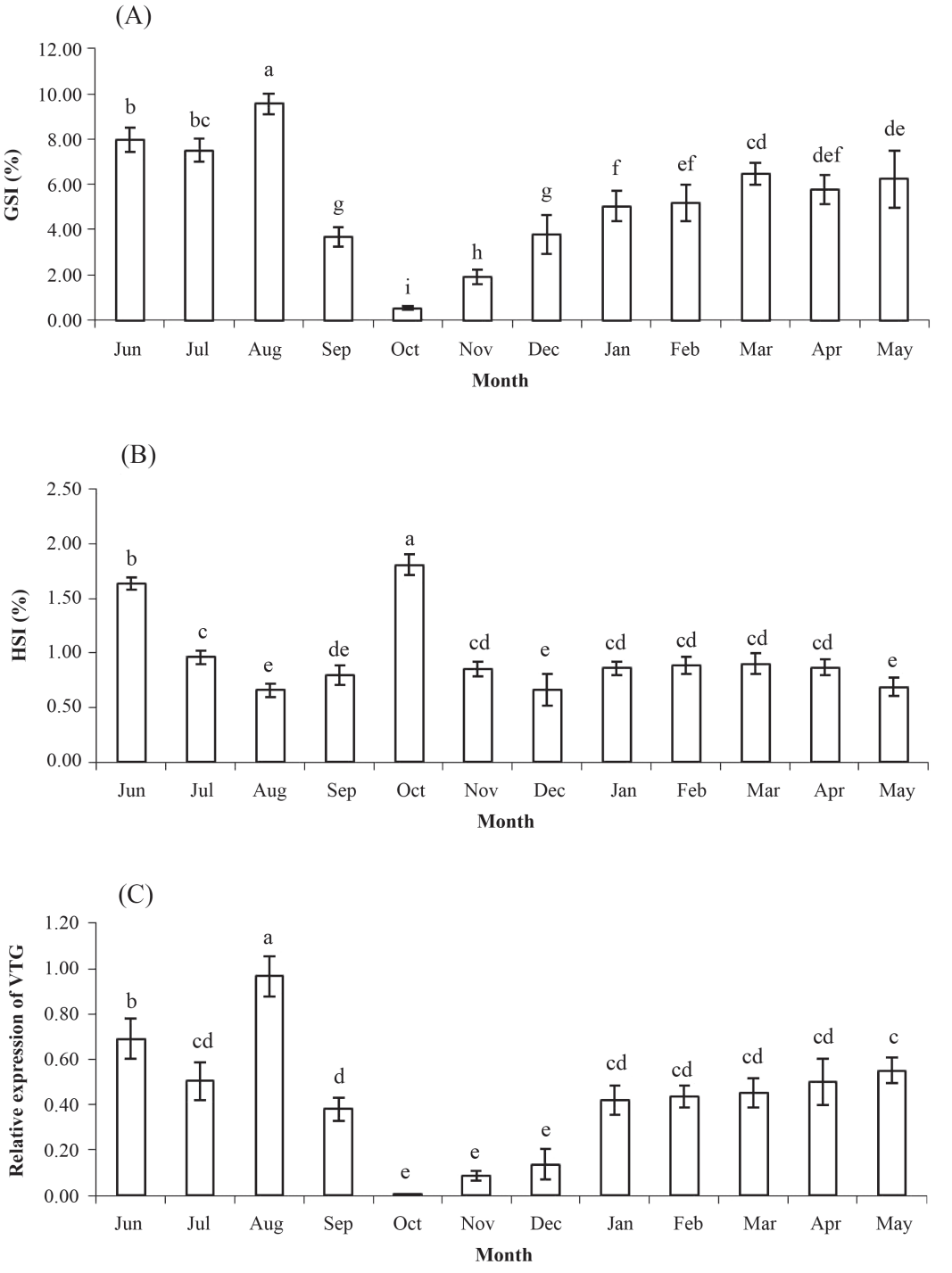

Figure 2. Mean values of gonadosomatic index (A), hepatosomatic index (B) and relative expression levels of vitellogenin gene (C) in *N. notopterus* (n = 6 in each month) in Kwan Phayao. Different letters indicate statistically significant differences ($P<0.05$).

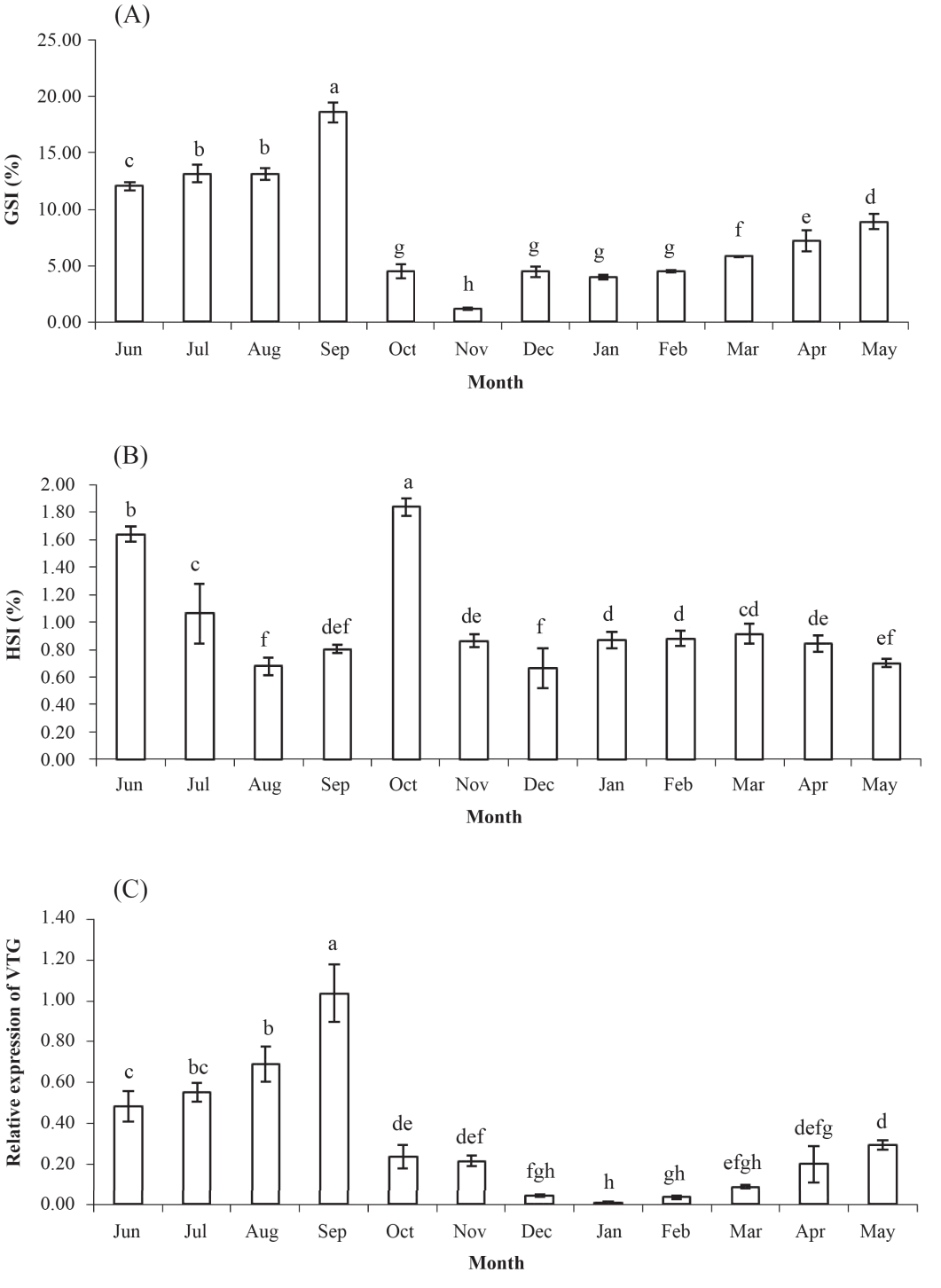

Figure 3. Mean values of gonadosomatic index (A), hepatosomatic index (B) and relative expression levels of vitellogenin gene (C) in *A. armatus* (n = 6 in each month) in Kwan Phayao. Different letters indicate statistically significant differences (P<0.05).

Table 3. Correlation analysis of GSI values, expression levels of VTG genes and HSI values in *N. notopterus* and *A. armatus*.

Fish species	GSI		
N. notopterus	**r = 0.91**	**VTG expression**	
	P<0.01		
A. armatus	**r_S = 0.82**		
	P<0.01		
N. notopterus	r = -0.25	r = -0.21	**HSI**
	NSD	NSD	
A. armatus	r_S = -0.12	r_S = -0.06	
	NSD	NSD	

Pearson and Spearman correlation coefficients are shown in r and r_S, respectively. Significant correlations are shown in bold and no significant differences are marked with NSD.

in both fishes, *N. notopterus* (r = 0.91; P< 0.01) and *A. armatus* (r_S = 0.82; P<0.01). While the relationships between the GSI values and expression levels of VTG gene with the HSI values tended to be inversely. However, there were no significant differences (P>0.01). The GSI values and expression levels of VTG genes simultaneous increased in the spawning period and decreased immediately after laying eggs. In contrast, the HSI values decreased in the spawning season and increased in the resting period.

DISCUSSION

Vitellogenins have been classified into three major types: types I, II and III, according to zebrafish VTGs (Wang *et al*., 2000), and each type is composed of two major yolk proteins, lipovitellin (LV) and phosvitin (PV) (Wallace, 1985). The lipovitellin consists of two polypeptides, a heavy chain (LVI) and a light chain (LVII). VTG type III is the shortest in length because it lacks phosvitin. In comparison with zebrafish VTGs, the *N. notopterus* and *A. armatus* VTGs are types I and II, respectively. Although the VTGs are of different types, these partial nucleotide sequences were cloned at the same domain following the position of primers, light chain lipovitellin, as lipovitellin is the predominant lipoprotein and is involved in lipid storage (Thompson and Banaszak, 2002). Therefore, lipovitellin is an important nutritional source of amino acids and lipids that support embryonic development (Patiño and Sullivan, 2002).

A phylogenetic tree was constructed from the different types of teleost fish VTGs to determine the evolutionary relationship. The nodes were clearly divided into three major branches based on the VTG types, VTG type I, II and III. The VTG type III was separated from the others because of lacking the phosvitin domain (Wang *et al*., 2000). This analysis confirmed that the fish VTGs composed of at least three types.

The reproductive cycles of *N. notopterus* and *A. armatus* in Kwan Phayao

were determined by performing monthly measurements of the mean GSI value and the expression of VTG in fish samples. The gonadosomatic index is typically used to evaluate the spawning season of fish. In addition, the GSI is a good indicator of gonadal development in fish (Dadzie and Wangila, 1980). In this study, the fish samples were of the mature size. Size at first maturity of *N. notopterus* and *A. armatus* were 65.5 (Jantharachit and Nuangsit, 2008) and 13.0 g (Nuangsit and Chansri, 2008), respectively. The GSI value of *N. notopterus* increased from November to August and peaked in August (9.57 ± 0.45%). This result showed that the spawning season of *N. notopterus* in Kwan Phayao occurred during this period. In a previous study, Jantharachit and Nuangsit (2008) reported that the spawning season of this fish species in Bung Lahan, Chaiyaphum province, occurred from February to September, nearly the same as in this study. In addition, the GSI value peaked in May (4.70%) with the gravid stage of maturity, followed by the spawning stage in July. However, the highest GSI value was less than that in this study by approximately two-fold, due to the different size (80.23 ± 36.86 g) of the fish. Moreover, the different environmental conditions in Kwan Phayao and Bung Lahan also affected GSI values. Numerous factors like size of fish (Dadzie and Wangila, 1980), age and the variations in culture conditions (Panprommin *et al*., 2008) correlated with the GSI values.

The spawning season of *A. armatus* in Kwan Phayao was from March to September, with the peak occurring in September (18.58 ± 0.62%). This result was supported by a previous study. Nuangsit and Chansri (2008) reported that the mean GSI value of this fish species increased from April to September in Lamtakong reservoir, Nakhon Ratchasima province, and was highest in September (16.64 ± 6.35%).

Vitellogenins are egg yolk precursor proteins that are normally synthesized by the liver tissue of female fish. Thus, the expression levels of the VTG gene should increase during the spawning season and decline in the spent stage. In this study, the expression levels of *N. notopterus* and *A. armatus* VTGs also increased in the spawning season, from January to August and March to September, respectively. These results showed that the expression of VTG is directly related to the GSI value. The expression profiles of VTG reflected the annual changes in the reproductive cycle of female fish, showing high levels during the breeding period and low levels during resting periods (Panprommin *et al*., 2008) according to the developmental stages of oocytes (Mikawa *et al*., 2006).

In the present study, the HSI of both *N. notopterus* and *A. armatus* are low in the spawning season (August). The HSI presented an inverse trend to the GSI. During spawning season, HSI decreases due to allocation of the energy to the gonads for oocyte development, and the GSI increases. The HSI is an indirect estimate of the energy status of an organism (Gomes *et al*., 2011). Furthermore, the HSI also involves the storage of reserves and the mobilization of energetic reserves for vitellogenesis and the reproduction process (Gomes *et al*., 2011).

The Department of Fisheries declares that the spawning season of fish is between

May 16 and September 15 every year for all water resources in Thailand, according to the Fisheries Act, B.E. 2490. During this time, no person is allowed to use any fishing appliance or any other methods in any freshwater fishery to allow the number of aquatic animals to recover. Because each water resource has a different environment, the spawning season of fish may be different. From this study, the GSI values and the VTG mRNA expression levels of *N. notopterus* and *A. armatus* showed that their spawning seasons are similar to the period declared by the Department of Fisheries. However, the spawning seasons of other fish in Kwan Phayao should also be confirmed.

CONCLUSION

This study is the first report of the VTG sequences of *N. notopterus* and *A. armatus*. The profiles of the VTG gene expression in annual changes of these fishes have a direct correlation with the GSI value, which is a good indicator of sexual maturation. Thus, the expression of VTG gene can be used to determine the spawning season of fish. However, both techniques, namely GSI value and hepatic VTG gene expression, are causing fish mortality. Further studies are required to develop methods to determine the spawning season using live fish samples, such as measuring the VTG gene expression in the blood.

ACKNOWLEDGEMENT

This study was funded by the National Research Council of Thailand (NRCT) under the project, "Fishery resources management in Kwan Phayao to achieve sustainable utilizing conservation", and was partially supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE).

LITERATURE CITED

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. **J. Mol. Biol.** 215: 403-410.

Andreassen, T.K., K. Skjoedt and B. Korsgaard. 2005. Upregulation of estrogen receptor α and vitellogenin in eelpout (*Zoarces viviparus*) by waterborne exposure to 4-*tert*-octylphenol and 17β-estradiol. **Comp. Biochem. Physiol.** C 140: 340-346.

Barucca, M., A. Canapa, E. Olmo and F. Regoli. 2006. Analysis of vitellogenin gene induction as a valuable biomarker of estrogenic exposure in various Mediterranean fish species. **Environ. Res.** 101: 68-73.

Dadzie, S. and B.C.C. Wangila. 1980. Reproductive biology, length-weight relationship and relative condition of pond raised *Tilapia zilli* (Gervais). **J. Fish Biol.** 17: 243-253.

Gomes, I.D., F.G. AraÚjo, W. Uehara and A. Sales. 2011. Reproductive biology of the armoured catfish *Loricariichthys castaneus* (Castelnau, 1855) in Lajes reservoir, southeastern Brazil. **J. Appl. Ichthyol.** 27: 1322-1331.

Htun-Han, M. 1978. The reproductive biology of the dab *Limanda limanda* (L.) in the North Sea: gonosomatic index, hepatosomatic index and condition factor. **J. Fish Biol.** 13: 369-378.

Jantharachit, P. and S. Nuangsit. 2008. Some biological aspects of grey feather back, *Notopterus notopterus* (Pallas, 1780) in Bung Lahan, Chaiyaphum province. **Technical paper** no. 45. Inland Fisheries Research and Development Bureau, Department of Fisheries. 47 pp. (*In Thai*).

Johnsen, H.K., H. Tveiten, N.P. Willassen and A.M. Arnesen. 1999. Arctic charr (*Salvelinus alpinus*) vitellogenin: development and validation of an enzyme -linked immunosorbent assay. **Comp. Biochem. Physiol.** 124B: 355-362.

Jones, P.D., W.M. De Coen, L. Tremblay and J.P. Glesy. 2000. Vitellogenin as a biomarker for environmental estrogens. **Water Sci. Technol.** 42: 1-14.

Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta Ct) method. **Methods** 25: 402-408.

Mikawa, N., T. Utoh, N. Horie, A. Okamura, Y. Yamada, A. Akazawa, S. Tanaka, K. Tsukamoto, I. Hirono and T. Aoki. 2006. Cloning and characterization of vitellogenin cDNA from the common Japanese conger *Conger myriaster* and vitellogenin gene expression during ovarian development. **Comp. Biochem. Physiol. B** 143(4): 404-414.

Mouchel, N., V. Trichet, A. Betz, J.P.L. Pennec and J. Wolff. 1996. Characterization of vitellogenin from rainbow trout (*Oncorhynchus mykiss*). **Gene** 174 (1): 59-64

Nuangsit, S. and P. Chansri. 2008. Reproductive biology of some fishes in Lamtakong reservoir, Nakhon Ratchasima province. **Technical paper** no. 22. Inland Fisheries Research and Development Bureau, Department of Fisheries. 70 pp. (*In Thai*).

Okumura, H., T. Todo, S. Adachi and K. Yamauchi. 2002. Changes in hepatic vitellogenin mRNA levels during oocyte development in the Japanese eel, *Anguilla japonica*. **Gen. Comp. Endocrinol.** 125(1): 9-16.

Orlando, E.F., G.A. Binczik, N.D. Denslow and L.J. Guillette. 2007. Reproductive seasonality of the female Florida gar, *Lepisosteus platyrhincus*. **Gen. Comp. Endocrinol.** 151(3): 318-324.

Panprommin, D., S. Poompuang and P. Srisapoome. 2008. Molecular characterization and seasonal expression of the vitellogenin gene from Günther's walking catfish *Clarias macrocephalus*. **Aquaculture** 276: 60-68.

Patiño, R. and C.V. Sullivan. 2002. Ovarian follicle growth, maturation, and ovulation in teleost fish. **Fish Physiol. Biochem.** 26(1): 57-70.

Sapbamrer, R. and S. Hongsibsong. 2014. Organophosphorus pesticide residues in vegetables from farms, markets, and a supermarket around Kwan Phayao Lake of northern Thailand. **Arch. Environ. Contam. Toxicol.** Doi 10.1007/s00244-014-0014-x

Scholz, S. and H.O. Gutzeit. 2000. 17-α-ethinylestradiol affects reproduction, sexual differentation and aromatase gene expression of the medaka (*Oryzias latipes*). **Aquat. Toxicol.** 50: 363-373.

Tamura, K., J. Dudley, M. Nei and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. **Mol. Biol. Evol.** 24: 1596-1599.

Thompson, J.D., D.G. Higgins and T.J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. **Nucleic Acids Res.** 22: 4673-4680.

Thompson, J.R. and L.J. Banaszak. 2002. Lipid -protein interactions in lipovitellin. **Biochemistry** 41: 9398-9409.

Tupwongse, V., P. Parkpian, P. Watcharasit and J. Satayavivad. 2007. Determination of levels of Mn, As, and other metals in water, sediment, and biota from Phayao lake, northern Thailand, and assessment of dietary exposure. **J Environ Sci Health A Tox Hazard Subst Environ Eng** 42(8): 1029-1041.

Wallace, R.A. 1985. **Vitellogenesis and oocyte growth in nonmammalian vertebrates.** In: Browder, L.W. (Ed.), Developmental Biology. Plenum Press, New York, pp. 127-177.

Wang, H., T. Yan, J.T. Tan and Z. Gong. 2000. A zebrafish vitellogenin gene (vg3) encodes a novel vitellogenin without a phosvitin domain and may represent a primitive vertebrate vitellogenin gene. **Gene** 256: 303-310.

Whangchai, N., S. Wanno, R. Gutierrez, K. Kannika, R. Promna, N. Iwami and T. Itayama. 2013. Accumulation of microcystins in water and economic fish in Phayao lake, and fish ponds along the Ing river tributary in Chiang Rai, Thailand. **Agricultural Sciences** 4(5B): 52-56.

Zhao, Y., L. Xie and Y. Yan. 2014. Microcystin -LR impairs zebrafish reproduction by affecting oogenesis and endocrine system. **Chemosphere** 120C: 115-122.