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Genetics and Biotechnologies for Sustainable Aquaculture of the Black Tiger Shrimp *Penaeus monodon*

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Abstract

The black tiger shrimp *Penaeus monodon* is one of the most important cultivated shrimp species. Reduced spawning potential and low degree of maturation of *P. monodon* in captivity crucially prohibit several possible applications including development of effective breeding programs of this species. Determining molecular mechanisms involving female broodstock quality (i.e. maturation potential) can be applied to promote reproductive performance of domesticated stocks. Accordingly, the use of molecular genetic markers that allow selection of broodstock with a high potential for reproductive success would be useful for the shrimp industry. In addition, the lack of high quality broodstock of *P. monodon* has been proposed to cause wide size variation of cultured shrimp at harvest. Thus, genetic improvements for increasing growth rate and size uniformity are key breeding objectives for this species. However, the fundamental controls of growth in penaeid shrimp are poorly understood. Identification of molecular markers that allow selection of juveniles and broodstock with high growth rates are being sought to increase the efficiency of genetic improvement in *P. monodon*. Here, the basic research about identification and characterization of genes/proteins differentially expressed in different stages of ovarian development of *P. monodon* is illustrated. Further studies for development of single nucleotide polymorphism (SNP) markers associated with reproduction and/or growth of domesticated *P. monodon* are discussed.

Keywords: Black tiger shrimp; *Penaeus monodon*; Reproduction; Growth; Genes; Proteins

1. Introduction

Farming of the black tiger shrimp (*Penaeus monodon*) has been practiced for food and the livelihood of coastal people in several Asian countries including Indonesia, the Philippines, Taiwan, Vietnam and Thailand for several decades. Initially, *P. monodon* was originally harvested together with other shrimp species from extensive growing ponds. In Thailand, the

first success in breeding of *P. monodon* at the Phuket Fisheries Station was in 1972, led to the commercial establishment of extensive and semi-intensive farms in 1972 and 1974, respectively. Subsequently, the ability to develop formulated feed commercially led to the success development of the intensive culture system of this species (FAO, 2005).

Reduced reproductive maturation of captive *P. monodon* females is found (Kenway et al., 2006; Preechaphol et al., 2007). Accordingly, breeding of pond-reared *P. monodon* is extremely difficult and rarely produced enough quality of larvae required by the industry. In Thailand, farming of *P. monodon* relies almost entirely on wild-caught broodstock for supply of juveniles (Klinbunga et al., 2001; Withyachumnarnkul et al., 1998). The lack of high quality wild and domesticated broodstock has probably caused the reduction of aquacultural production of *P. monodon* since the last several years (Limsuwan, 2004).

Eyestalk ablation is used commercially to induce the maturation of penaeid shrimp ovaries, but the technique leads to eventually lose in egg quality and death from disease infectious of the spawner (Benzie, 1998). Hence, predictable maturation and spawning of captive *P. monodon* without the use of unilateral eyestalk technique is an ultimate goal for the shrimp industry (Prechaphol et al., 2007; Quackenbush, 2001).

The domestication and selective breeding programs of this species would provide a more reliable supply of juveniles and the improvement of their production efficiency. The use of selectively bred stocks having improved culture performances on commercially desired traits rather than the reliance on wild-caught stocks is a major mean of sustainability of the shrimp industry (Benzie, 1998; Clifford and Preston, 2006; Coman et al., 2006). Nevertheless, genetic improvement of *P. monodon* is slow owing to the lack of the basic information related with ovarian development and maturation in penaeid shrimp.

In addition, information on the relationships between genotypic and phenotypic variations of shrimp is limited at present. Analysis of gene-based single nucleotide polymorphisms (SNPs) is one of the efficiently reliable approaches for discovery of genes that significantly contribute in production traits of this commercially important species (Liu and Cordes, 2004; Tao and Boulding, 2003). This allows the possibility to identify major loci responsible for the difference in quantitative traits of *P. monodon* where this information is not currently available in this species (Klinbunga et al., 2009 and 2015). The use of genetic markers that allow selection of broodstock with a high potential for reproductive success would be useful for the shrimp production industry.

Here, examples of research on isolation and expression analysis of reproduction-related genes are illustrated. Additional studies on the discovery of SNPs in reproduction-related genes and evaluation of the association between identified SNPs and reproduction parameters (e.g. ovarian weight and gonadosomatic index, GSI) in broodstock are shown. In addition, the association between functionally important genes and growth parameters (average body weight and total length) of *P. monodon* juveniles is also demonstrated.

2. Isolation and expression analysis of reproduction-related genes and protein during ovarian development of *P. monodon*.

Ovarian maturation of *P. monodon* results from rapid synthesis and accumulation of a major yolk protein, vitellin which is derived from its precursor, vitellogenin. In penaeid shrimp, vitellogenin (Vtg) is synthesized in both hepatopancreas and ovaries, transported to oocytes and accumulated in the ooplasm as vitellin for utilization as a nutritional source during embryogenesis (Fainzilber et al., 1992; Kawazoe et al., 2000; Tiu et al., 2006; Yano, 1995).

The levels of hemolymph Vtg protein increase during ovarian development of penaeid shrimp (Meusy and Payen, 1988; Okumura et al., 2006; Tiu et al., 2006; Yano et al., 1996).

Neuropeptides that directly affect reproductive performances in crustacean have been identified. Gonad inhibiting hormone (GIH) which is secreted from the X-organ in the eyestalk, and inhibits the synthesis of vitellogenin in the ovary (Huberman, 2000). Treerattrakool et al. (2008) cloned and characterized GIH from cDNA obtained from the eyestalk of *P. monodon* (*Pem*-GIH). Tissue expression analysis was performed. A knockdown experiment of *Pem*-GIH was carried out using dsRNA interference. The *Pem*-GIH protein contained 79 amino acids and was closely related to type II crustacean hyperglycemic hormone (CHH). The *Pem*-GIH gene expression was observed in eyestalk, brain, thoracic and abdominal nerve cords of adult *P. monodon*. Injection of dsRNA of *Pem*-GIH can reduce transcript levels in the eyestalk and in the abdominal nerve cord both *in vitro* and *in vivo*. *Pem*-GIH-knockdown shrimp showed an increase in expression level of vitellogenin mRNA.

Peptides with gonad inhibiting properties were also characterized in Pacific white shrimp *Litopenaeus vannamei* (Tsutsui et al., 2007) and American lobster *Homarus americanus* (called VIH; Ohira et al., 2006). Both GIH from *L. vannamei* and *H. americanus* have shown an *in vitro* effect to inhibit the expression of the *Vtg* gene.

For transcriptomic analysis, a typical ovarian cDNA library was established for isolation of genes involving ovarian (and oocyte) development in *P. monodon*. EST analysis was carried out against 2330 clones and results indicated that *PmVtg1* was expressed only in the ovarian cDNA library but not in those established from other tissues of *P. monodon*. Although *P. monodon Vtg* protein is reported to be present in hepatopancreas (Avarre et al., 2003; Raviv et al., 2006; Tiu et al., 2006; Tsang et al., 2003; Tsutsui et al., 2005). EST representing *PmVtg1* was not observed in the hepatopancreas library after 5419 clones were sequenced because the library was originally established from juvenile shrimp (Tassanakajon et al., 2006; http://pmonodon.biotec.or.th).

Subsequently, the expression profiles and localization of *PmVtg1* were examined. *PmVtg1* mRNA was expressed only in ovaries and hepatopancreas but not in other tissues of female and testes of male broodstock. In wild intact broodstock, *PmVtg1* mRNA was expressed at a low level in stage I ovaries and up-regulated in stages II (vitellogenic) and III (late vitellogenic) being significantly reduced in stage IV (mature) ovaries ($P < 0.05$). In eyestalk-ablated broodstock, the expression level of *PmVtg1* mRNA was increased to nearly a peak level in vitellogenic (stage II) ovaries and peaked in late vitellogenic (stage III) ovaries ($P < 0.05$). The level of *PmVtg1* mRNA in hepatopancreas of intact shrimp with stages II and III ovaries was significantly greater than those with stages IV (mature) and V (post-spawning) ovaries. Interestingly, *PmVtg1* mRNA in hepatopancreas was 25-40 times higher than that in ovaries of intact shrimp with stages I-III of ovarian development. *In situ* hybridization revealed that *PmVtg1* mRNA was clearly localized in the cytoplasm of follicular cells surrounding late previtellogenic, vitellogenic and cortical rod oocytes (Hiransuchalert et al., 2013a).

There has been no evidence on the autosynthesis of *Vtg* mRNA within oocytes of penaeid shrimp. Quantitative real-time PCR against different periods of spawned eggs suggested that *PmVtg1* mRNA was also synthesized in oocytes. Western blot analysis revealed a similar profile of ovarian *P. monodon* vitellin and *PmVtg1* mRNA during ovarian development. Immunohistochemistry confirmed that Pm-vitellin protein was localized in developing, vitellogenic and mature oocytes but not in the follicular cells. The information suggested that *PmVtg1* mRNA and Pm-vitellin protein play the main role in development of oocytes and ovaries in *P. monodon*. The basic knowledge obtained allows further characterization of

mechanisms of vitellogenesis in this economically important species. (Hiransuchalert et al., 2013a).

Methyl farnesoate (MF) is the crustacean homolog of the insect juvenile hormone (JH III) and is believed to regulate growth and reproduction in crustaceans (Huberman, 2000). It has been reported that MF maintains juvenile morphology and, therefore, inhibits gonadal development in juveniles but enhances reproductive maturation in adults (Borst and Laufer, 1990; Laufer and Sagi, 1991; Laufer et al., 1998). MF is synthesized in mandibular organ (MO) from farnesoic acid (FA) by the action of farnesoic acid O-methyltransferase (FAMeT) in the presence of *S*-adenosyl methionine (Nagaraju, 2007).

In *P. monodon*, two full-length cDNAs of *PmFAMeT* were identified. They were 1296 and 1311 bp in length containing ORFs of 828 (*PmFAMeT-s*) and 843 (*PmFAMeT-l*) bp deducing to polypeptides of 275 and 280 amino acids. *PmFAMeT* mRNA was down-regulated at stages II (vitellogenic) and III (late vitellogenic) ovaries and up-regulated in stage IV (mature) ovaries in wild intact broodstock ($P < 0.05$). In eyestalk-ablated broodstock, its expression level in stages III and IV ovaries was significantly greater than that in stages I and II ovaries ($P < 0.05$). Exogenous serotonin injection (5-HT; 50 μg/g body weight) significantly promoted its expression level in ovaries of 18-month-old shrimp approximately 50 folds at 1 h post injection (hpi, $P < 0.05$; Fig. 1). *PmFAMeT* mRNA was localized in previtellogenic oocytes but was not observed in oogonia and more mature stages of oocytes. Western blot analysis revealed the positively reactive band of 32 kDa along with a smaller band of approximately 29 kDa in stages I-IV ovaries of wild intact broodstock. The latter was not observed in premature ovaries of juveniles (Buaklin et al., 2015).

Cyclin-dependent kinases (Cdks) plays primary role in eukaryotic cell cycle progression. Cdk subunits with their cyclins subunit typically involves in different stages of cell cycle progression (Honda et al., 1993). The activity of Cdks depends on the association with cyclins and is regulated by phosphorylation on certain key tyrosine and threonine residues

The meiotic maturation of animal oocytes is controlled by the maturation promoting factor (MPF; Okano-Uchida et al., 1998; Kishimoto 2003 and 2018). In most species, cytoplasmic MPF is maintained in an inactive form (called pre-MPF) by inhibitory phosphorylation of Cdc2 at Thr14 and Tyr15 by Myt1 kinase and at Thr161 by cyclin-activating kinase (CAK), a complex of cyclin-dependent kinase 7 (Cdk7)/cyclin H or Cdk7/cyclin H/Mat 1 (Harper and Elledge, 1998; Patel and Simon, 2010; Tassan et al., 1995). Dephosphorylation of Thr14 and Tyr15 residues of Cdc2 by Cdc25 phosphatase leads to the resumption of meiotic maturation of oocytes (Clarke et al., 1992; Dunphy et al., 1988; Dunphy and Kumagai, 1991; Jessus et al., 1991; Mueller, et al., 1995). Alternatively, a different mechanism of oocyte resumption has been reported in some amphibians and fishes where Cdc2 presents as a monomer with no phosphorylation due to the absence of cyclin B in immature oocytes. Only Thr161 phosphorylation by CAK is required for MPF activation (Hirai et al., 1992; Honda et al., 1993; Kobayashi et al., 1991; Yamashita et al., 1995).

In *P. monodon*, the full-length cDNA of *PmCdc2* was formerly identified by EST analysis of the testis cDNA library (Leelatanawit et al., 2009). The complete coding sequence of *PmCdc2* from ovaries was also characterized (Phinyo et al., 2013) and its ORF length was identical with that of *PmCdc2* from testes. The conserved phosphorylation sites of Thr14, Tyr15 and Thr161 residues were found in both transcripts. The potential *N*-linked glycosylation site was not found in the deduced PmCdc2 protein. The catalytic domain of serine/threonine kinases (S_TKc) was found at positions 4-287 of the deduced PmCdc2 protein. The PSTAIRE (positions 45-51) and DFG (positions 146-148) which are related with the cyclin binding, and

GxGxxGxV (GEGTYGVV, positions 11-18) elements which is involved in ATP binding (De Bondt et al., 1993) were also found.

In addition, the full-length cDNA of ovarian *PmCdk7* was also characterized (Phinyo et al., 2014). Its deduced amino acid sequence was identical to that previously identified in testes (Leelatanawit et al., 2009). Two potential polyadenylation signal sequences (AATAAA) were found in *PmCdk7* suggesting the possible selective polyadenylation usage. The T-loop (positions 164-191) which is a region of major conformational difference between active and inactive forms was found in the deduced PmCdc7 protein as in other Cdk proteins of *P. monodon*, such as PmCdc2 (DFGLARAFGIPVRVYTHEVVTLWYRAPE located at positions 146-173, accession no. EU492538; Phinyo et al., 2013). T-loop phosphorylation favors a kinase conformation which allows the access of substrate to the active site (Morgan and De Bondt, 1994; Taylor et al., 1992). Cdk7 is unusual among Cdks because dual phosphorylation in the T-loop (e.g. at S170 and T176 in *Xenopus* Cdk7 and positions Ser164 and Thr170 in *Drosophila* Cdk7 is required for its activation (Martinez et al., 1997; Larochelle et al., 2001). Therefore, the actual positions for activating phosphorylation of PmCdk7 should be further determined.

3. Studies on induction of reproduction-related genes by eyestalk ablation and/or exogenous injection of serotonin

Understanding the induction mechanisms of reproduction-related genes will be useful in developing effective methodologies for induction of ovarian maturation in *P. monodon* (Ibara et al., 2007). Eyestalk ablation resulted in an increased mRNA level of vitellogenin in ovaries of penaeid shrimp (Hiransuchalert et al., 2013a; Okumura et al., 2006; Tsutsui et al., 2005). As a result, expression of reproduction-related genes induced by eyestalk ablation and/or exogenous neurotransmitter or steroid hormone administration should reflect the progression in reproductive maturation of female *P. monodon* adults.

Our previous studies clearly showed that unilateral eyestalk ablation promotes the expression of several reproduction-related genes, including *farnesoic acid O-methyltransferase* (*PmFAMeT*), an important enzyme that converts farnesoic acid to methyfarnesoate, in ovarian stage III (Buaklin et al., 2015), *progestin membrane receptor component 1* (*PmPgmrc1*), a key gene in the progesterone inducing pathway, in ovarian stages II-IV (Preechaphol et al., 2010), *mitogen-activating protein kinase* during the final maturation of ovaries (*PmMAPK1*; Ponza et al., 2011), *cell division cycle 2* (*PmCdc2*) and *cell-dependent kinase cycle 7* (*PmCdk7*), important genes for signal transduction during oocyte meiotic maturation in ovarian stages I-IV (Phinyo et al., 2013 and 2014). In addition, the expression levels of *small androgen receptor-interacting protein* (*PmSARIP*; Hiransuchalert et al., 2013b) and *bystin 1* (*PmBys1*; Phinyo et al., 2018) were also induced by eyestalk ablation.

Serotonin (5-HT) has an indirect impact on reproductive maturation by influencing the release of relevant hormones or by modulating the responses of the target tissues to the hormones. It has a wide range of effects on many physiological responses such as pigment regulation, energy metabolism, osmoregulation, and ion balance (Chang et al., 2007; Fingerman 1997; Sathyanandam et al., 2008). Effects of exogenous serotonin (5-HT) injection on the reproductive performance and maturation of *Procambarus clarkii*, *Homarus americanus* (Fingerman, 1997; Kulkarni et al., 1992), *Macrobrachium rosenbergii* (Meeratana et al., 2006), *Litopenaeus vannamei* (Alfaro et al., 2004; Vaca and Alfaro, 2000), *Penaeus semisulcatus* (Aktas and Kumlu, 2005), *Fenneropenaeus merguiensis* (Makkapan et al., 2011) and *P. monodon* (Wongprasert et al., 2006) were reported.

Our studies revealed that 5-HT administration clearly promoted expression of *P. monodon Ovarian-Specific Transcript* (*Pm-OST1*) in ovaries of subadults (5-month-old) at 12-78 hours post injection (hpi), with the peak level at 48 hpi (Klinbunga et al., 2009). In addition, its effects on promoting the expression of *P. monodon adipose differentiation related protein* (*PmADRP*) in ovaries of subadult shrimp at 48 hpi were also reported (Sittikankaew et al., 2010).

In adults, 18-month-old domesticated shrimp were injected with 5-HT (50 µg/g body weight) and it clearly promoted the expression level of *small androgen receptor-interacting protein 1* (*PmSARIP1*; Hiransuchalert et al., 2013b), *PmFAMeT* (Buaklin et al., 2015), *bystin 1* (*PmBys1*; Phinyo et al., 2018), *Pm17β-hydroxysteroid dehydrogenase* (*Pm17β-HSD*; Treejate, 2011), *PmCdc2* (Phinyo et al., 2013) and *PmCdk7* (Phinyo et al., 2014) (Fig. 1A-F; $P < 0.05$). Likewise, its injection resulted in up-regulation of *GTP binding protein alpha subunit Go* (*PmG$_{\alpha o}$*) which is functionally important in the meiotic signal transduction pathway of oocytes, at 6-12 hpi (Yocawibun, 2011).

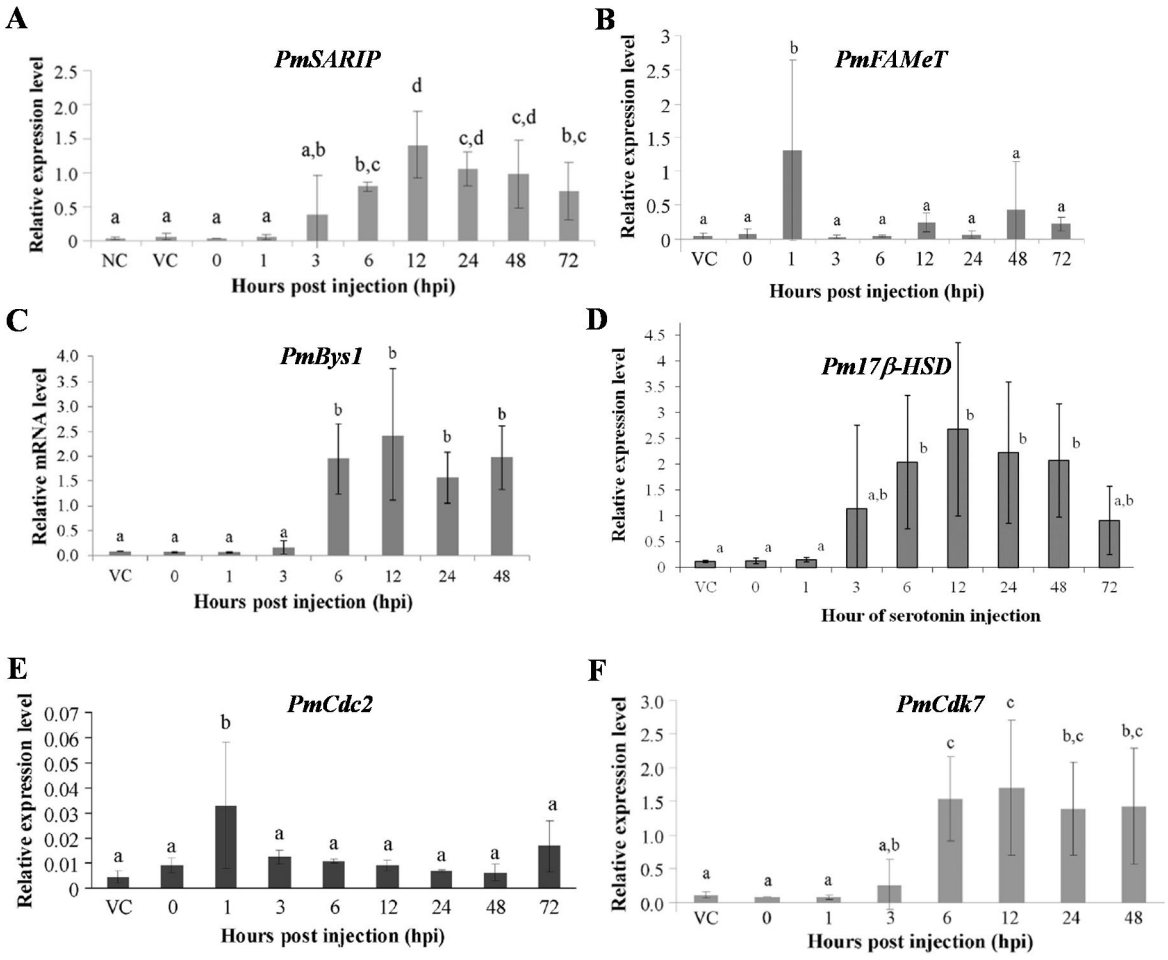

Fig. 1 Mean relative expression levels of *PmSARIP* (A), *PmFAMeT* (B), *PmBys1* (C), *Pm17β-HSD* (D), *PmCdc2* (E) and *PmCdk7* (F) in ovaries of domesticated 18-month-old shrimp injected with 5-HT and assessed at 0, 1, 3, 6, 12, 24, 48 (C and F) and 72 (A-B and D-E) hpi ($N = 4$ for each group). Bars labeled with the same letters above the histograms reveal non-significant differences between groups of samples ($P > 0.05$). VC = shrimp injected with 0.85% NaCl at 0 hpi (vehicle control).

Effects of progesterone injection on expression of reproduction-related genes were also examined. Interestingly, only *PmPgmrc1* and *PmCytb5* transcripts which encode proteins containing one transmembrane domain and a cytochrome b5 domain, were induced by exogenous progesterone injection (0.1 μg/g body weight in 14-month-old shrimp; Fig. 2A and 2B). Nevertheless, the expression of other genes described above was not significantly affected by progesterone injection.

Results from expression analysis of various reproduction-related genes upon injection with 5-HT suggest that molecular activities of 5-HT were similar to those from eyestalk ablation. This implied its effects on induction of gene expression through a membrane receptor with the contribution of heterotrimeric G-proteins. The signal stimulus results in the induced transcription of genes in various pathways (e.g. methyl farnesoate, steroid biosynthesis and signal transduction pathways). Subsequently, 5-HT may directly enhance meiotic maturation of oocytes in *P. monodon* by stimulation of the MPF (a complex of Cdc2 and Cyclin B protein) via the activity of CDK (PmCdk7 protein and its partners, Cyclin H and MAT1 which have still not been identified and characterized in *P. monodon*).

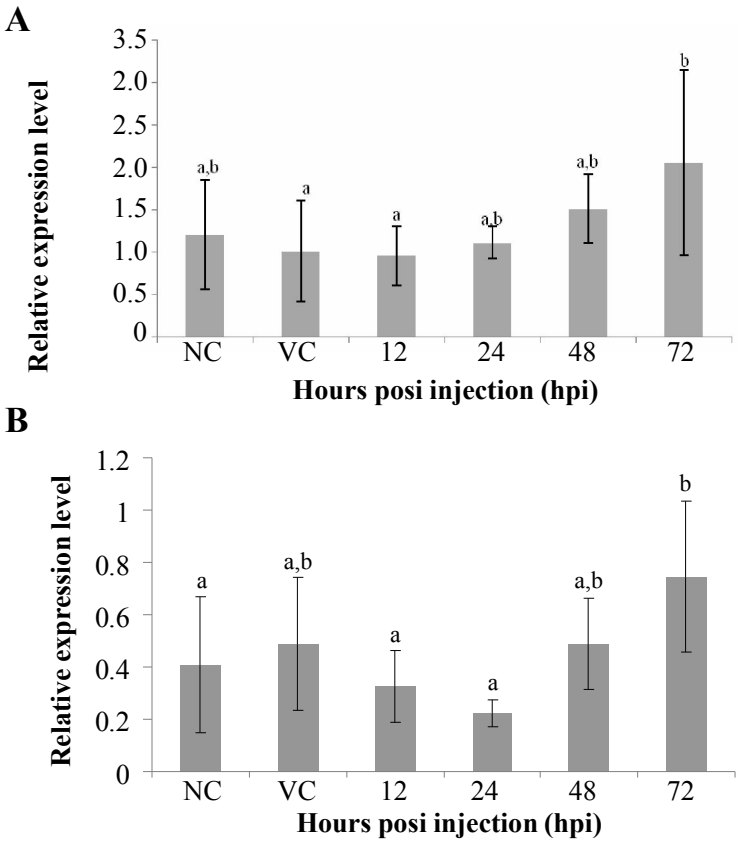

Fig. 2 Time-course relative expression levels of *PmPgmrc1* (A) and *PmCytb5* (B) in ovaries of domesticated 14-month-old broodstock at 12, 24, 48 and 72 hpi of progesterone (0.1 μg/g body weight). Shrimp injected with absolute ethanol were included as the vehicle control (VC). Acclimated shrimp without any treatment were included as the negative control (NC). The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Therefore, appropriate concentrations of 5-HT on induction of ovarian development of a particular stage of domesticated *P. monodon* should be carefully examined to implement the possible use of this neurotransmitter for enhancing ovarian development in *P. monodon* in place of eyestalk ablation.

4. Studies on gene/protein functionally involved with ovarian development and association between SNPs and reproduction and/or growth of *P. monodon*

Genetic improvement of *P. monodon* is slow due to the lack of the basic information related with its ovarian development and maturation. Determining molecular mechanisms involving female broodstock quality (i.e. maturation potential) can be applied to promote reproductive performance of domesticated stocks. In addition, the primary goal of genetic selection is the growth improvement of economically important species. Molecular markers that allow for selection of juveniles and broodstock with a good performance for reproductive maturation and/or growth rates are useful and can be applied to improve selection processes of *P. monodon* (Jung et al., 2013; Klinbunga et al., 2015).

The *P. monodon vitellogenin receptor* (*PmVtgr*) was first characterized by Tiu et al. (2008). The expression level of ovarian *Vtgr* mRNA examined by Northern blot analysis was low during early vitellogenesis and peaked in late vitellogenesis. Immunohistochemistry revealed the positive signal of the Vtgr protein in plasma membrane of developing oocytes. Injection of dsRNA of VtgR resulted in a decrease in Vtgr protein content in ovaries, but an increase of Vtg levels in the hemolymph. However, association between SNPs of functionally important genes and reproduction-related parameters (e.g. gonadosomatic index, GSI and/or ovarian weight) in *P. monodon* has not been reported.

In our recent study, the full-length cDNA (5990 bp in length with an ORF of 5832 bp deducing to a polypeptide of 1943 amino acids) and expression levels of *PmVtgr* during ovarian development in wild shrimp and association between its SNPs and reproduction-related parameters in domesticated shrimp were examined. *PmVtgr* was only expressed in ovaries but not in other tissues of female broodstock. The expression level of *PmVtgr* transcript in premature ovaries of juveniles was significantly lower than in those of broodstock ($P < 0.05$). In intact broodstock, *PmVtgr* mRNA significantly increased in stage IV (mature) relative to stage I (previtellogenic) ovaries ($P < 0.05$). However, *PmVtgr* mRNA was comparably expressed at all stages of ovarian development in eyestalk-ablated shrimp ($P < 0.05$; Klinbunga et al., 2015).

In addition, SNPs in the *PmVtgr* gene segment of 14-month-old *P. monodon* ($N = 64$) were indirectly examined by single-stranded conformational polymorphism (SSCP). Significant relationships between different SSCP patterns of the *PmVtgr* gene and GSI and ovarian weight of *P. monodon* were found. Results were consistent when further tested against non-related 19-month-old shrimp ($N = 54$, $P < 0.05$). Different SSCP genotypes of the *PmVtgr* gene segment from 14-month-old shrimp were cloned and sequenced ($N = 10$ for each SSCP pattern). SNP positions 126 (C→T), 226 (T→A), 441 (T→A), 477 (T → A), 499 (A → C), 500 (T → C), 519 (A → G), 540 (A → G), 545 (G → A), 614 (T → C) and 678 (A→C) of the *PmVtgr* gene segment were significantly associated with GSI values of examined shrimp ($N = 40$). Phenotype-associated SNP markers identified allow the possible application to assist genetic selection of *P. monodon* (Klinbunga et al., 2015).

To assess the possible biological roles of *PmFAMeT* and *catechol O-methyltransferase* (*PmCOMT*) and *X-box binding protein 1* (*PmXbp1*) in reproduction and growth of *P. monodon*, their cDNA sequences were characterized. Expression patterns of these transcripts during

ovarian development in wild *P. monodon* broodstock were examined (Fig. 3). Effects of eyestalk ablation (wild broodstock) and serotonin induction (domesticated broodstock) on expression levels of ovarian *PmFAMeT*, *PmCOMT* and *PmXbp1* mRNAs were examined. In addition, analysis of relationships between and expression levels of *PmFAMeT* and reproductive traits in adults or that of *PmXbp1* and growth traits in juveniles was performed (Buaklin et al., 2015 and 2016; Prasertlux et al., 2015).

SNPs in the *PmFAMeT* gene segment of 14-month-old *P. monodon* ($N = 65$) were indirectly examined by SSCP analysis. Results revealed that domesticated shrimp having different SSCP (Orita et al., 1989) genotypes of *PmFAMeT* possessed a different average ovarian weight ($P < 0.05$). Six SNPs were found in cloned *PmFAMeT* gene segments of 14-month-old shrimp ($N = 65$). Two SNPs (positions c.487+g.10 which refer to a SNP located in an intron at 10 nucleotides after the cDNA position 487 and c.487+g.174+c.38 which refers to a SNP located in an exon at 38 nucleotides after the cDNA position 487 + an intron of 174 bp relative to the *PmFAMeT* gene) were significantly associated with an average ovarian weight of examined shrimp. The relative expression level of ovarian *PmFAMeT* mRNA in shrimp with different genotypes of these SNPs was also significantly different ($P < 0.001$, Fig. 4A and 4B; $P < 0.05$).

In addition, association between SNP genotypes and growth parameters of 5-month-old domesticated juveniles was examined. Analysis of its nucleotide sequence revealed that SNP at positions c.487+g.10 and c.487+g.174+c.8 (refers to a SNP located in an exon at 8 nucleotides after the cDNA position 487 + an intron of 174 bp relative to the *PmFAMeT* gene) can be restricted with *HpyCH4V*. Interestingly, two positions of SNPs are found, as a result, the predicted genotypes could be deduced for either each locus or from both positions as composite SNP genotypes (diplotypes). PCR-RFLP was carried out against genomic DNA of 23 full-sib families of the 7th generation of domesticated *P. monodon* cultured in the concrete ponds ($N = 323$). At position c.487+g.10, significant differences between growth parameters of juveniles having different genotypes were observed where juveniles with a $T/C_{c.487+g.10}$ genotype showed a greater average body weight and total length than those with the $T/T_{c.487+g.10}$ and $C/C_{c.487+g.10}$ genotype ($P < 0.05$). At position c.487+g.174+c.8, significant differences between the average body weight of juveniles having different genotpyes were also observed where juveniles with $A/A_{c.487+g.174+c.8}$ and $A/G_{c.487+g.174+c.8}$ had a greater average body weight and total length than those with $G/G_{c.487+g.174+c.8}$ ($P < 0.001$). Similarly, significant differences in growth-related parameters were observed in juveniles carrying different *PmFAMeT* diplotypes ($P < 0.001$) (Rongmung et al., 2017).

Subsequently, *PmCOMT* cDNA and gene were characterized (Buaklin et al., 2016). Genomic sequence of *PmCOMT* spanned 1466 bp in length containing 3 exons (194, 111 and 361 bp) and 2 introns (143 and 147 bp). The full-length cDNA of *PmCOMT* was 1176 bp in length with an ORF of 666 bp corresponding to a polypeptide of 221 amino acids. *PmCOMT* mRNA was not differentially expressed during ovarian development in wild intact *P. monodon* adults ($P > 0.05$). Eyestalk ablation resulted in up-regulation of *PmCOMT* in stages II (vitellogenic) and IV (mature) relative to stage I (previtellogenic) ovaries ($P < 0.05$; Fig. 3B). In domesticated shrimp, its expression was reduced in 18-month-old adults compared with 6-month-old juveniles and 14-month-old adults ($P < 0.05$).

Recombinant PmCOMT protein and anti-rPmCOMT polyclonal antibody were successfully produced. The PmCOMT protein was localized in ooplasm of previtellogenic oocytes. It was temporally found in both ooplasm and nucleus of vitellogenic oocytes and subsequently in cortical rods (Crs) and the Cr membrane of mature oocytes. The information on protein

expression and localization further suggest that PmCOMT protein is a multifunctional protein and may also be functionally involved in fertilization and embryogenesis of *P. monodon*.

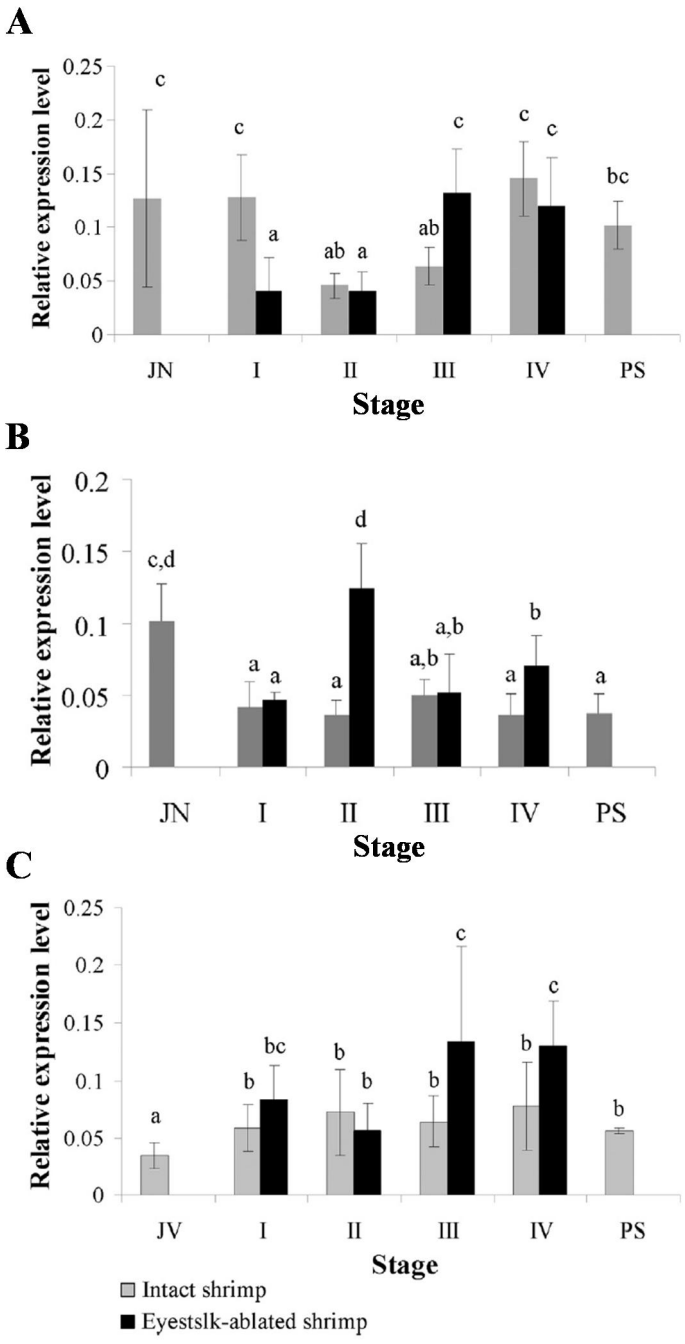

Fig. 3 Relative expression levels of *PmFAMeT* (A), *PmCOMT* (B) and *PmXbp1* (C) mRNAs during ovarian maturation of wild intact and unilateral eyestalk-ablated *P. monodon* adults. The levels were measured as an absolute copy number of *PmFAMeT*, *PmCOMT* or *PmXbp1* and normalized by that of *EF-1α.* Each histogram corresponds to different stages of ovaries. The bars correspond to the standard deviation (SD) of the means. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$). JN = juvenile ovaries; I-IV = previtellogenic, vitellogenic, late vitellogenic, and mature ovaries, respectively; PS = ovaries of intact adults immediately collected after spawning (stage V).

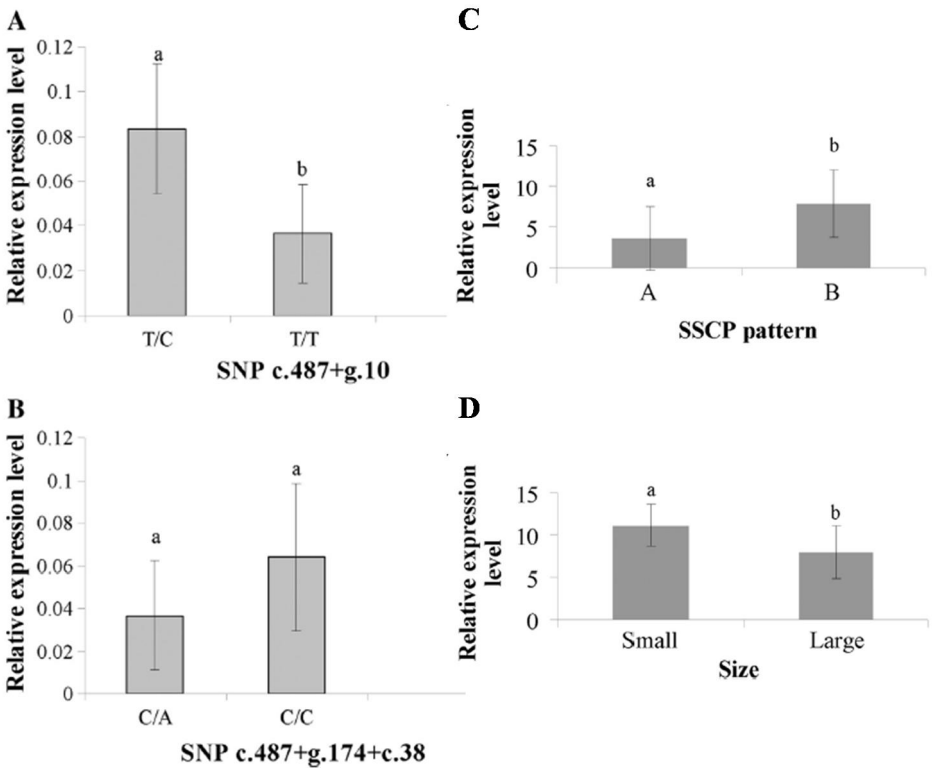

Fig. 4 Histograms illustrating the relative expression level of *PmFAMeT* in 14-month-old domesticated broodstock exhibiting different SNP genotypes at positions c.487+g.10 (A) and c.487+c.174+g.38 (B) of *PmFAMeT*. In addition, relationships between the relative expression level of *PmXbp1* in hepatopancreas of 3-month-old *P. monodon* juveniles having different SSCP genotypes ($N = 44$, C) and growth performance according to the body weight ($N = 19$, D) are shown. The bars correspond to standard deviation (SD) of the means. The same letters above the bars reveal non-significant differences between groups of samples ($P > 0.05$).

In addition, SNP in the *PmCOMT* gene segment of domesticated *P. monodon* (14-month-old shrimp, $N = 64$) was identified by SSCP and PCR-direct sequencing. Two SSCP patterns (A and B) corresponding to $G/C_{c.305 + g.84}$ and $C/C_{c.305 + g.84}$ SNP genotypes were found. Shrimp carrying the former genotype (OW = 0.92 ± 0.37 g and GSI = 1.06 ± 0.39%, $N = 31$) had a greater average ovarian weight and GSI value than those carrying the latter genotype (OW = 0.75 ± 0.30 g and GSI = 0.86 ± 0.32%, $N = 33$) ($P < 0.05$).

Moreover, the full-length cDNA of *PmXbp1* in *P. monodon* was characterized. It was 1762 bp in length containing an ORF of 855 bp corresponding to 284 amino acids. The expression level of *PmXbp1* in different ovarian stages of wild intact broodstock was comparable but greater than that of cultured juveniles ($P < 0.05$). Eyestalk ablation resulted in up-regulation of *PmXbp1* in stages III (late vitellogenic) and IV (mature) ovaries of wild *P. monodon* broodstock ($P < 0.05$; Fig. 3C). Exogenous serotonin injection (5-HT, 50 µg/g body weight) potentially promoted the expression of *PmXbp1* in ovaries of domesticated 18-month-old broodstock at 24 hpi ($P < 0.05$).

SNPs in *PmXbp1* (185 bp) were examined in domesticated 3-month-old juveniles (average body weight and total length = 12.32 ± 5.13 g and 11.30 ± 1.58 cm, $N = 162$). Association between SSCP patterns of *PmXbp1* and growth-related parameters was found where the

average body weight (15.07 ± 5.04, $N = 44$) of juveniles carrying pattern A (corresponding to a T/T_{349} SNP of the amplified *PmXbp1* fragment) was significantly greater than that (11.37 ± 4.80, $N = 116$) of juveniles carrying pattern B (corresponding to a T/C_{349} SNP) (Fig. 4C). The relative expression levels of *PmXbp1* in the hepatopancreas of juveniles carrying different SSCP/SNP genotypes were significantly different (A > B; $P < 0.05$; Fig. 4C). In addition, the level of *PmXbp1* in shrimp exhibiting a greater growth performance (22.08 ± 2.97 g and 13.60 ± 0.56 cm; $N = 11$) was significantly lower than that with a lesser growth performance (5.00 ± 0.89 g and 8.60 ± 0.98 cm; $N = 8$) ($P < 0.05$; Fig. 4D) (Prasertlux et al., 2015).

Taken the information together, *PmFAMeT*, *PmCOMT* and *PmXbp1* should play the functional roles during ovarian development of *P. monodon* (Buaklin et al., 2015 and 2016; Prasertlux et al., 2015). Moreover, *PmFAMeT* and *PmXbp1* seem to play an important role in reproduction and growth of *P. monodon*. Significant association between SNPs identified in several genes and growth parameters of domesticated *P. monodon* was found. The identified markers could be applied for selection of domesticated broodstock for breeding of the next generation shrimp.

In conclusion, eyestalk ablation is commonly used to induce ovarian maturation of penaeid shrimp in captivity. Molecular mechanisms of the ablation were studied and revealed that this technique promotes the expression levels of genes encoding proteins in vitellogenesis, signal transduction, methylfarnesoate and steroid biosynthesis pathways. Similar induction effects were found from serotonin administration. These findings shed light on molecular mechanisms of ovarian development and maturation of *P. monodon* leading to an ultimate goal of finding alternative methods to replace the undesirable practice of the eyestalk ablation in the future.Acknowledgments

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Cage Culture of Monosex Tilapia for Food and Financial Security

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Abstract

Cage culture is becoming common in canals, rivers, reservoirs and lakes mainly with the booming monosex tilapia farming. Monosex-male tilapia has several advantages i.e. it grows fast, does not breed, and can be stocked at high densities. More importantly, tilapia meat has no Y-bones, which makes it suitable for making fillets for export. In Thailand, most farmers use standard size of cages either 5 m x 5 m or 6 m x 4 m with 2 m in depth. They stock about 2,000 juveniles (30-40g)/cage. Farmers harvest about 1 ton of fish in 5-6 months with the size ranging from 0.6 to 1 kg and provide approximately US$1,000 net profit/crop. Tilapia cage culture technology is rapidly expanded to other countries such as Bangladesh where about 4,000 cages were installed during 2005-2011. Recently, India starts cage culture in lakes and reservoirs. Therefore, it is going to be a big boom for tilapia cage culture in the area. In Vietnam, cage culture of tilapia is also expanding. In Indonesia, Malaysia, Zambia, and Zimbabwe, tilapia is grown in large-circular cages used by salmon farms. The cages often reach 20 m in diameter and the production can be up to 100 tons/cage. Some countries are still skeptical in allowing full-fledge cage culture e.g. Nepal where fish are still not allowed to be fed with pellets as it was thought that this will pollute the water. This paper presents recent developments and contribution of cage culture to food security, and also discusses about environmental issues, which are required, as cage culture will be expected because aquaculture production needs to be double by 2050.

Keywords: Monosex tilapia; Cage culture; Food Security; Environment

1. Introduction

Demand for seafood is soaring with rapidly growing middle class and increased awareness about its health benefits but seafood supply is dwindling due to decline in wild stock. Based on increment trend in per capita fish, aquaculture production has to be double by 2050 from its current production of 80 million tons (Zhou, 2018). However, land, water and feed ingredients to support the growth are limited. Asia will be the one to support this growth. Annual world per capita meat consumption stands at 46.5 kg whereas fish consumption is only about 20 kg/capita, which is way below the levels of other unhealthy meats. Europeans consume over 40 kg pork/year as compared to 23 kg fish. People are aware that more fish should be consumed, as it is the healthiest protein. Therefore, anytime soon demand may soar which was indicated in a recent survey conducted by Cargill (Michaelson, 2018). Only its availability and affordability are the major problems. In SE Asia, fish has been traditionally the main source of animal protein. Per capita consumption reaches over 30 kg annually, whereas in South Asian

countries e.g. Nepal and Pakistan it is only about 2 kg. One-third animal protein is required in the regular diet for good health (AIT, 1994), but it reaches hardly 10%, resulting in malnutrition and stunting, especially in women and children. Such issue is quite high in Bangladesh (36.3%), India (47.7%), Nepal (34.1%) and Pakistan (45.5%) and it is more intense in rural areas with low dietary diversity (Bhujel et al., 2008; Krishna et al., 2017).

Asia produces nearly 90% of the farmed seafood in the world using many of the 300 cultured aquatic species (e.g. carps, tilapia, salmon, shrimp and *Pangasius*). Amongst them, tilapia is second only to carps. Unlike carps, its meat has no Y-bones, which makes it easier for consumption and also suitable for making fillets for export. It took over salmon in 2005 in terms of volume owing to the technology developed for the mass-scale production of monosex seed. It is more suitable for cage culture than carps as it performs well at high densities, utilizes natural foods, and tolerates a wide range of environmental conditions. Monosex-male tilapia, which can be both black and red, has been the most suitable type as it grows faster than normal tilapia as well as most others and does not breed in the system that minimizes the problem of fish escapees. Indeed, cage culture has expanded in terms of cage size, farming area and production volume considerably due to the use of monosex tilapia.

The most reliable method of monosex tilapia production is by using standard method of feeding male hormone for 21 days as described by Bhujel (2014). The method was developed as a package by AIT and commercialized. Using the technology, several hatcheries in Thailand supply over 10 million high quality fry per month (Bhujel, 2010 and 2011). There are probably hundreds of monosex hatcheries but largest five hatcheries produce already over a billion fry per year.

In Thailand, only about 3% farmers are using cages out of 300,000 farmers growing tilapia but they produce well over 30% by volume (Belton et al., 2009). Cage culture is expanding rapidly to many countries, along with monosex seed production technology as quality seed is abundantly available to the farmers. Cage culture along with monosex tilapia has significant contribution to food security, employment and income generation especially for rural communities. It could play greater role, if the water resources such as canals, lakes, reservoirs, streams and rivers are allowed to utilize. However, environmental concerns associated with some commercial aquaculture practices for higher income and export are over-shadowing the need for the rural communities. This paper is a review and compilation of information on current practices of cage culture of monosex tilapia. Data or information were obtained from various sources including direct field observations, interactions with farmers, and also workshop and discussions with several groups (http://aarm-asialink.info/activities.html) from various countries who came to AIT to receive short-term trainings and join study tours. It gives a brief overview of recent advances in cage aquaculture of monosex tilapia in Asia and beyond, and emphasizes its potential to draw attention of all stakeholders including policy makers.

2. Brief country reports

2.1 Thailand

Mass scale production of monosex tilapia fry has contributed to expand cage culture. Initially, Nile tilapia (*Oreochromis niloticus*) was introduced from Japan to Thailand as Royal Gift in 1965 (Pullin, 1988). After three decades of research and development, and active promotion, it became one of the most important fish species cultured in Thailand. Tilapia industry took off especially during the last two decades. About one thousand tilapia hatcheries have emerged in Thailand. Several of them can supply over 20 million high quality monosex fry each month (Bhujel, 2014).

Cage culture was further boosted due to monosex red tilapia initiated by CP Co. Ltd. Thailand. The company gave a new and distinct name i.e. Thabthim (means ruby) attractive to the local communities. Branding along with marketing efforts has boosted its demand and so the cage culture in many countries of SE Asia. Depending upon the interest, red and black tilapias are grown in standard cages (5 m x 5 m or 6 m x 4 m with 2 m in depth installed in rivers, canals or lakes (Fig. 1). Although red tilapia cage farmers are fewer, they contribute up to 30-50% of the total tilapia production (Belton et al., 2009). Farmers feed at 5-8% of the biomass/day of good quality floating pellets containing around 30-35% crude proteins twice or thrice daily. They stock 30-40 g size and harvest about 1 ton fish per cage (i.e. 20 kg /m^3 of 0.6-1 kg size). Farming is becoming specialized and segmented which is the sign of maturity. Net profit is about US$1,000 per cage. Similar systems are also found in Cambodia, Laos and Vietnam along the Mekong River and its tributaries.

Fig. 1 Tilapia grown in cages (6 m x 4 m) in Mekong River in Nong Khai Province, Thailand. (Photo by Ram Bhujel).

2.2 Bangladesh

Several attempts of tilapia introduction were made without success. A government official trained at AIT transferred the monosex hatchery technology to private sector due to which millions of monosex fry production was made possible (Baqui and Bhujel, 2011). After the success of monosex seed production, tilapia became a booming industry (Baqui and Bhujel, 2011). Cage culture became popular in rivers in Chandpur and Lakhipur where over 6,000 tilapia cages (Pervez, 2018); including landless people supported by various organizations. Most cages are of 6 m x 3 m x 1.5 m. They stock 20 g fingerlings at 37-40 fish per m^3 and harvest at about 400 g size but few times. They feed floating pellets, FCR of which is about 1.75 and productivity is 350 kg per cage i.e. 13 kg per m^3. Profitability shows about US$ 200 per cage per 6 months.

2.3 Vietnam

Vietnamese *Pangasius* farmers are facing tough competition; therefore, many of them are now shifting to monosex tilapia farming. Existing good infrastructure can easily handle up to 2.8 million tones fillet per year but only half of its capacity has been used for *Pangasius*. It can then be used for tilapia industry. Vietnam increased tilapia exports by 76%, 142% and 265%

during 2012 to 2014, respectively. Many cage farms are operated along the Mekong River. Some of the largest farms have over 100 cages. Each cage is 16 m x 8 m x 4 m (512 m^3) in size. With the stocking densities at about 80-100 fish per m^3, they produce about 20 ton per cage i.e. 39 kg per m^3. The country has many rivers and long coastline from North to South, which can be utilized for cage culture. The Ministry of Agriculture and Rural Development (MARD) has recently passed the Development Scheme on large-scale tilapia cultivation by 2020 with a vision towards 2030 (VOV, 2016). According to the scheme, they plan for 1.5 million m^3 cage volume on rivers and reservoirs by 2020 and 1.8 million m^3 cage by 2030 to create over 50,000 jobs with the target productions of 0.3 million MT and 0.4 million MT by 2020 and 2030, respectively. However, the main obstacle for tilapia industry is shortage of high quality monosex seed. There are about 200 small tilapia hatcheries producing about 0.5 billion per year but still large quantity of broodstocks and monosex fry are being imported.

2.4 The Philippines

A large amount of tilapia is produced in cages especially in Laguna and Taal lakes. Sizes of cages range from small to large (e.g. 10 m x 10 m with the depth of 3-4 m). Millions of monosex fingerlings used to be produced by large hatcheries but many of them have collapsed due to natural disasters and other reasons. Similarly, large amounts of tilapias were produced in cages in those lakes without feeding in the past. Nowadays, they need to be fed. Use of excessive number of cages caused fish kills and creased environmental concerns.

2.5 India

Until 2014 tilapia was not allowed for commercial farming formally in India because of the bad image of *O. mossambicus*. However, the benefits of tilapia in terms of taste and potential export market were realized, Private sectors were interested to diversify the aquaculture from traditional carps, which have no chance to export. Due to pressure from the private sector and international efforts, some state governments started considering tilapia farming such as Andhra Pradesh, Kerala, Karnataka and Tamil Nadu. Tilapia farming is starting to boom and some being exported. More recently, state governments are giving their cage culture facilities to the private sector to utilize for monosex tilapia and *Pangasius* farming. Large increase on the tilapia farming is therefore expected soon.

2.6 Other countries

Similarly, tilapia farming is expanding rapidly in other countries in Asia (e.g. Cambodia Laos, Pakistan and Nepal) and South Pacific Island nations (e.g. Fiji, Papua New Guinea, and so on). Indonesia ranks second in tilapia production in the world after China with an approximate production of 1 million ton annually for which nearly half of the production is produced in cages and floating nets. Monosex tilapia has not been widely accepted yet in China, the largest producer of tilapia (1.7 million MT/year). Monosex tilapia culture could bring the big change if promoted by the government and private sector.

In Cambodia and Laos, monosex tilapia was promoted by Aqua Outreach of AIT since early 1990s. Laos was one of the major tilapia producing countries in the world, which produces over 30,000 MT annually in ponds and cages along the Mekong River. The cages are similar to the Thai models and most of seed is imported from Thailand. Recently, there are several AIT model hatcheries producing monosex fry carried out. The main problem in Laos is low temperature.

In Cambodia, the Government promotes tilapia in rural areas only which are away from natural water bodies which predominates the fish production. The government is reluctant to

promote tilapia cage culture due to availability of wild fish especially in Mekong and the Great Lake, Tonle Sap.

Pakistan is already starting cage culture of monosex tilapia in some lakes and reservoirs. If irrigation canals and more rivers, reservoirs and lakes could be utilized, increased fish production would help reduce malnutrition and stunting in children.

In Nepal, cage culture of carps has been practiced since 1970s in lakes and reservoirs. There are few hatcheries starting to produce monosex tilapia. It would be more suitable for culture to increase the cage productivity and profitability.

Malaysia has high demand for tilapia. Red tilapia production has recently increased replacing black tilapia. Good market acceptance in domestic markets and also availability of high quality fry (mostly imported) and commercial feed are the major contributing factors. Private and public agencies are promoting monosex tilapia farming in ponds and cages

Among Sub-Saharan African countries (e.g. Ghana, Ivory Coast, Nigeria, Uganda, Zambia and Zimbabwe), some of them have large tilapia cage farms and produce considerable amount of tilapia. However, the availability of quality monosex seed and broodstock is questionable.

In Latin America, cage culture of monosex tilapia is just starting only in Brazil and Mexico. Due to proximity to the US market, tilapia farming in cages is aggressively promoted.

3. Main lessons and concerns

3.1 Monosex tilapia

Cage culture is expanding rapidly with the emergence of monosex tilapia hatcheries. In the past, cage culture was mostly done using carps at low stocking densities. Cage farmers had difficulty in making profit since it took a long culture period to get marketable size (>1 kg) as they grow slowly. Monosex tilapia has provided good alternative. It grows faster and can be stocked at 5-10 times higher densities. More importantly, monosex tilapia would not breed in the cages if they get converted applying right methods and protocols. Owing to this reason, India and Pakistan are allowing tilapia to be introduced and cage culture to be done in natural water bodies where tilapia was banned officially in the past.

3.2 Cage size/volume

Shape and size of cages varied widely. In case of tilapia, cage evolved from small size cages to bigger as production needs to be increased due to the growing demand for fish. However, small or low-volume high-density (LVHD) cages promoted by American Soybean Association (ASA) have high productivity per unit volume of water (Zhou et al., 2012). Stocking of up to 300 fish/m^3 of 50 g in size has been successful to produce 500 g fish in about 4 months. Culture of fish in these LVHD cages (2 m x 2 m x 1 m) was good to supply food for small families. However, 10,000 smaller cages (2 m x 1.3 m x 1.9 m) were earlier promoted in Bangladesh to support poor families involving women during 1990s (Roy et al., 1996). Nevertheless, almost all disappeared after the project period ended. There might be about 500 cages left but they are used mainly for nursing of fry and fingerlings, instead of grow-out.

There are several reasons behind this but the main reason might be the low volume of fish and revenue. To increase production volume, farmers have to add more numbers of cages. However, it adds to the cost for cage construction and management; for example, feeding in 10 small cages takes about 10 times more efforts instead of in a large cage. To take economy of scale, cage sizes are enlarging, and larger size cages become more common as farmers could produce and earn significant incomes by selling surplus fish. The cages of 5 m x 5 m x 2 m or

6 m x 3 m x 2 m cages became very popular. Larger cages are found in Vietnam (16 m x 8 m x 4 m) and the Philippines (10 m x 10 m x 3 m). More recently, farmers use bigger cages in Indonesia, Malaysia, Malawi, Zambia and Zimbabwe with the circular sizes of 19-24 m in diameter originally designed for salmon. They produce 35-65 tons of fish per cage when stocked with 150,000 or more fingerlings. The major underlying reasons behind adopting the larger cages are the necessity to increase production volume and to take advantage of economy of scale.

3.3 Role of private sector

Cage culture of tilapia expanded so rapidly mainly due to promotion by private sector with a view to selling feed and seed. In addition, private sector did branding of its product. For example, the CP Co. Ltd. in Thailand branded red tilapia as "Thapthim" has contracts with groups of cage farmers which also buys back their fish for post-harvest handling and processing or export. All the farms using large circular cages belong to the corporate entities rather than family managed farming. This indicates that farmers need input and marketing supports in addition to technical information in order to carry out cage culture.

3.4 Hormone use

Due to lack of scientific understanding, there are some concerns about the use of methyl testosterone (MT) to produce monosex tilapia. First of all, the hormone is manufactured for human being to use as medicine. The dose matters than the material. The hatcheries use only 60 mg/kg feed, which is so little compared to the dose (300 mg/day) prescribed by medical doctors for young boys to solve the problem of stunting. The one-day dose is used to produce 20,000-30,000 monosex fry of 0.2 g in size. The hormone does not remain in the body of fish. It comes out within a week after stopping the 21-day feeding period. Even if all hormone remained, a person would have to eat 20,000 fish to get that one-day dose which is impossible. Therefore, the use of MT to produce young monosex tilapia seed should be considered a safe technique as it is permitted by Food and Drug Administration (FDA) of USA (Smitherson, 1993).

3.5 Pollution from cages

Cage culture has been considered as polluter of water and the environment as nutrients are added in to the system. Nutrients leaching into the water can be quantified. For example, if we install 100 cages to produce about 100 tons of fish, and fish are fed 3-4% of biomass twice a day with the feed contains around 30% crude proteins and the protein contains about 16% nitrogen (N), and assuming 70% digestibility of feed, nutrients concentration can be simply estimated by dividing the volume of water body as shown below:

Amount of nitrogen expected to be in the environment
= 100 ton x 2.0 FCR x 30% undigested + leftover x 30% CP x 16% Nitrogen
= 2,880 kg N per 32 weeks of grow-out period
= 90 kg per week

If a reservoir is 100 ha in size, which means 1 km long and 1 km wide, the total volume of water would be:
= 100 ha x 10,000 m^2 x 1000 liter
= 1,000,000,000 litres of water volume

Increased nitrogen due to cage culture = 90 x 1,000 x 1,000 / (1,000,000,000 L)
= 0.09 ppm per week

The estimation shows that the increment in nitrogen level per week due to feeding fish in cages would be negligible amount. A recent study in Bangladesh (Hossain et al., 2017) has reported that the remaining feed is actually consumed by local fish outside the cages. Therefore, cage culture normally is not the real cause of pollution unless lakes or reservoirs are full of cages and fish are over-fed. It can also be argued that tilapia is filter feeding fish, thus it will take nutrients and help clean the water. Water pollution is mainly due to the run-off water especially from agricultural fields where pesticides are heavily used, and the wastes from industries and municipalities that are directly drained. However, having man-made structures in pristine water especially the lake, reservoirs, rivers or sea may create question of destroying natural beauty. In such cases, authorities should have a zoning so that cage culture can be located away from such touristic areas.

3.6 Cage culture in ponds

As the rivers, lakes and reservoirs where cages are installed belong to the community to use for transportation, irrigation and so on. In Thailand, two mass-scale fish kills occurred along Chaophraya and Bang Pakong Rivers. The fish kill in Chaophraya River was due to the molasses poured into the river when a large barge full of molasses was capsized. While in Bang Pakong River, fish kills were believed to be due to the excessive humus/acidic materials and pesticides from run-off water from surrounding agricultural land accumulated at the sluice gate, that was opened suddenly when rain started. Therefore, in order to avoid such risks, some farmers / companies started culturing in cages and hapa nets installed in large ponds (Fig. 2). They collect larger fish from the pond and stock in cages to feed at high rates. The uneaten food becomes food for the fish outside the cages in pond. Farmers normally grade them by size and stock in different cages so that they can sell a cage a day or week.

Fig. 2 Tilapia grown in cages (6 m x 4 m x 2 m) installed in ponds, Chiang Mai, Thailand and other fish are outside cages without feeding (Photo by Ram Bhujel).

3.7 Monosex tilapia in coastal and cooler areas

Mostly monosex tilapias are cultured in freshwaters. As red hybrid variety of tilapia can be grown at up to 30 ppt, it can be a good candidate for mariculture in brackish water and full strength seawaters. Some attempts of genetic research and acclimatization protocol have helped in this regard. Black Nile tilapias have been tried to acclimatize for up to 20 ppt. Therefore, monosex black Nile tilapia also has a potential for cage culture in brackish water areas. These are likely to be solutions to dwindling wild-caught sea fish. However, more research efforts are needed. Similarly, attempts have been made to develop cold tolerance so that they could be cultured in cages in cooler areas or during the winter season.

3.8 Feeds and feeding

Varying with countries, feed cost accounted for around 40-80% depending upon the farming system. In case of pond system, fertilization can reduce up to 50% of the feed cost, i.e. 40% of the total cost. However, fertilization is not possible for cage culture. Feed cost can be 60-80% of the total cost. Although attempts have been made to produce cheaper tilapia feed using minimal level of fishmeal (i.e. 3% or even zero %). Complete replacement has negative impacts on growth unless limited amino acids are supplemented. There is always a trade-off between good quality feed and the price. Thorough understanding is more important, so the farmers proper training, for the practical management of feeds and feeding to operate cage culture profitably.

3.9 Economics

For cage grow-out business, the main cost was for feed, which is about 60%, followed by cage construction (17%) and the seed (16%) (Ram Bhujel, unpublished data). Therefore, the farmers who can manage the feeds and feeding regime based on the understanding of biological aspects of the species, water quality and weather conditions, can take advantages of cage culture. It also depends on the market price of fish. A simplified estimation shows that there is a potential of getting US$500-1,000 per cage per cycle of 5-6 months from a standard cage of 5 m x 5 m or 6 m x 4 m with 2 m depth, which about 20-24% profit margin depending upon the price of fish.

4. Conclusions and recommendations

Monosex tilapia has enormously contributed to the expansion of cage culture thereby playing crucial roles in creating employment and making cheap but still high quality protein available to the people. Traditional cage culture has expanded from few community ponds, lakes and reservoirs to more on rivers, canals and some private ponds mainly. Monosex tilapia emerged as the most suitable tropical fish for cage culture because it does not waste energy in reproduction and grows fast at high densities and adverse environment.

Cages are getting bigger for economy of scale and to produce more fish to meet the high demand for fish. Along with the monosex hatchery technology, cage culture practice is spreading from Thailand to Bangladesh, Cambodia, Laos, India, Pakistan and other Asian countries including African continent during this and the last two decades.

Degree of technology adoption and production of monosex tilapia in cages vary with countries. Bangladesh and Vietnam are the foremost adopters and great beneficiaries, while India is just starting, but it is rapidly moving forward. Being adjacent to Thailand, Cambodia and Myanmar are lagging behind, most probably because they still have abundant wild fish available. More efforts are needed for them to take advantages of monosex technology of tilapia and its culture in cages to utilize their huge water resource. Firstly, policy makers have to be

clear that wild fish catch is not sustainable and aquaculture is the only way to feed the growing population by producing fish *en masse*.

High quality fish seed is the basic requirement and the proven monosex technology is available to produce required seed in a mass scale. However, people are still skeptical about the use of hormone to produce monosex tilapia. Similarly, policy makers and other stakeholders are also doubtful about the pollution and other environmental problems thought to be caused by cage culture. These require scientific explanation and clarification by the experts. Organizing seminars, workshops, interactions between experts and policy makers along with other stakeholders, training and study tours would be fruitful. Convincing them is a huge task. In addition to clarifying with facts, socio-economic benefits need to be highlighted. For example, cage culture does not require land. It can be done by landless people in public water bodies for which government can manage if they feel it is the need of the country. Successes of cage culture of monosex tilapia depend on the policy of the respective government, efforts of development agencies, NGOs and private sector. Awareness creation, transfer of technologies, access to market and inputs such as seed, feed and equipment, availability of required education, training and consultancy services to concerned authorities, farmers and their groups and entrepreneurs/investors play key roles. Proper zoning of aquaculture, practical education and research would be helpful.

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Impacts of Climate Change on the Tilapia Value Chain from Cage Culture in Luzon, Philippines

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Abstract

The impacts of major climate change hazards experienced by tilapia value chain players in Luzon, Philippines were analyzed. The impacts were estimated in terms of direct and indirect effects on the economic activities across key segments in the value chain. The direct effects involve either damage to operating inputs, fixed assets, and outputs or defensive expenditures incurred by players in trying to prevent or minimize the potential impacts or both. Indirect effects were captured through the cascade of impacts to other players from players directly affected, mainly through input and output prices. Using the value chain approach, the economic viability of cage culture activities as well as the associated risks along the tilapia chain were highlighted. Data were collected mainly through the interviews with key players in the production and trading nodes of the value chain, local government units, and Bureau of Fisheries and Aquatic Resources. Result pointed out that direct impacts were largest in the production segments of the value chain (i.e. input provision from ponds and production culture in cages). Under extreme weather conditions, losses among players manifested either through reduced volume in tilapia produced/traded or diminished value at times of excessive supply/harvest of smaller sized tilapia. While share of cage culture operators was the largest among chain players, they also bear the highest share in the risk and losses in the face of climate change hazards. Various adaptation strategies practiced by value chain players were also noted.

Keywords: Climate change impacts; Value chain; Tilapia cage culture; Adaptation

1. Introduction

The production of tilapia in the Philippines has dramatically tripled over the past two decades with a Luzon island group accounting for more than 90% of the annual tilapia harvest volume. In 2015, national tilapia production reached 261,210 metric tons (MT) and 92.87% of which were cultured in freshwater environment while the remaining 7.11% were in brackish waters (Table 1). Around 30% of the tilapia production consistently comes from cage culture in freshwater lakes in the Calabarzon region.

In the Calabarzon region, the province of Batangas contributed 58,723.54 MT accounting for 72.4% of the region's total production in 2015. Due to low survivability of tilapia from water temperature changes in Taal Lake, lesser harvests were recorded from January to March 2015. Late stocking due to lack of financial resources of fishers compounded the problem. In

2014, the production losses in the Calabarzon region were occurred from washed-out aquaculture structures, fish pens, and fish cages due to impacts from a super typhoon.

Historical accounts, data, and studies show an increase in the frequency and intensity of weather events in the Philippines. Stronger typhoons were identified as the primary climate-induced hazard affecting cage culture. In 2014, Typhoon Glenda, with international name Typhoon Rammasun is one of the most extreme climate change phenomena encountered by the tilapia industry in the Calabarzon region. The strong winds brought by Typhoon Glenda caused massive physical damage and even aggravated incidences of sulfur upwelling leading to fish kills. Data show a significant decrease in the tilapia production in Calabarzon region in 2014 due to lost production from washed-out aquaculture farm structures and fish cages.

Table 1 Philippine tilapia production by culture environment and system, 2006 & 2015

Culture environment and system	2006		2015	
	Production volume (MT)	**Share (%)**	**Production volume (MT)**	**Share (%)**
Brackish water	11,972.13	5.93	18,560.36	7.11
Freshwater	189,967.34	94.04	242,588.12	92.87
Pond	*114,045.34*	*56.45*	*142,339.01*	*54.49*
Pen	*15,619.75*	*7.73*	*21,460.25*	*8.22*
Cage	***60,302.25***	***29.85***	***78,788.86***	***30.16***
Rice-fish system	0	0.00	2.75	0.00
Small farm reservoir	75.67	0.04	59.22	0.02
National Production	**202,015.14**		**261,210.45**	

MT = metric tons
Source of basic data: PSA (2016)

Based on the report of the Taal Volcano Protected Landscape-Protected Areas Management Board, Typhoon Glenda caused damage to 2,168 out of 6,000 fish cages in the Taal Lake, with an estimated value of US$ 26.4 million worth of losses. In this context, the study estimated and analyzed the adverse impacts in terms of direct and indirect effects on the economic activities across key segments in the value chain.

2. Materials and Methods

Value chain involves the full range of activities necessary to bring a product or service from conception, through the different phases of production, delivery to final consumers, and final disposal after use (Hellin and Meijer 2006; Kaplinsky and Morris 2001). The fish value chain analysis framework used for considering impacts of climate change is shown by Fig. 1.

The flow of methodologies used is presented by Fig. 2. The first two steps provided an overview of the tilapia industry and existing climate-related hazards, respectively, through a rapid assessment carried out by organizing the available literature and secondary data. The next step was value chain mapping which was done by identifying the major chains in the tilapia industry and locating the key players at the most downstream end (i.e. exporter, processor, institutional buyer, big wholesaler or retailer) then trace the chain backward to the most upstream end (i.e. nursery and hatchery operators, feed suppliers). From the traders initially interviewed in the market, the grow-out operators and other key players in the value chain were

identified, located and interviewed. Central in the conducted key informant interviews (KIIs) was the estimation of the value-addition that each player has created at each stage of the value chain. Value-addition has been defined in this study as the difference between the sale of tilapia at each stage of the value chain and the accompanying cost of all resources used in the process. The fourth step estimated value-additions from each of the key players in the chain were using a simple cost and returns analysis. In the next step, impact of climate change on the chain players' activities and economic performance were estimated using "with and without" climate change hazards scenarios. The adaptation measures to neutralize the adverse effects of climate change on income and livelihood were also determined and defensive expenditures were estimated. Both the direct and indirect damages were identified and estimated. The former included the reduction in gross income, damages to fixed assets, and other incidental expenditures. The latter captured the reduction in traders' volume and frequency of operations because of lower harvests as offshoot of climate change hazards. The last step required the dissemination and validation of initial study results of the study through focus group discussions (FGDs) with participants that represented the different players of the tilapia value chain.

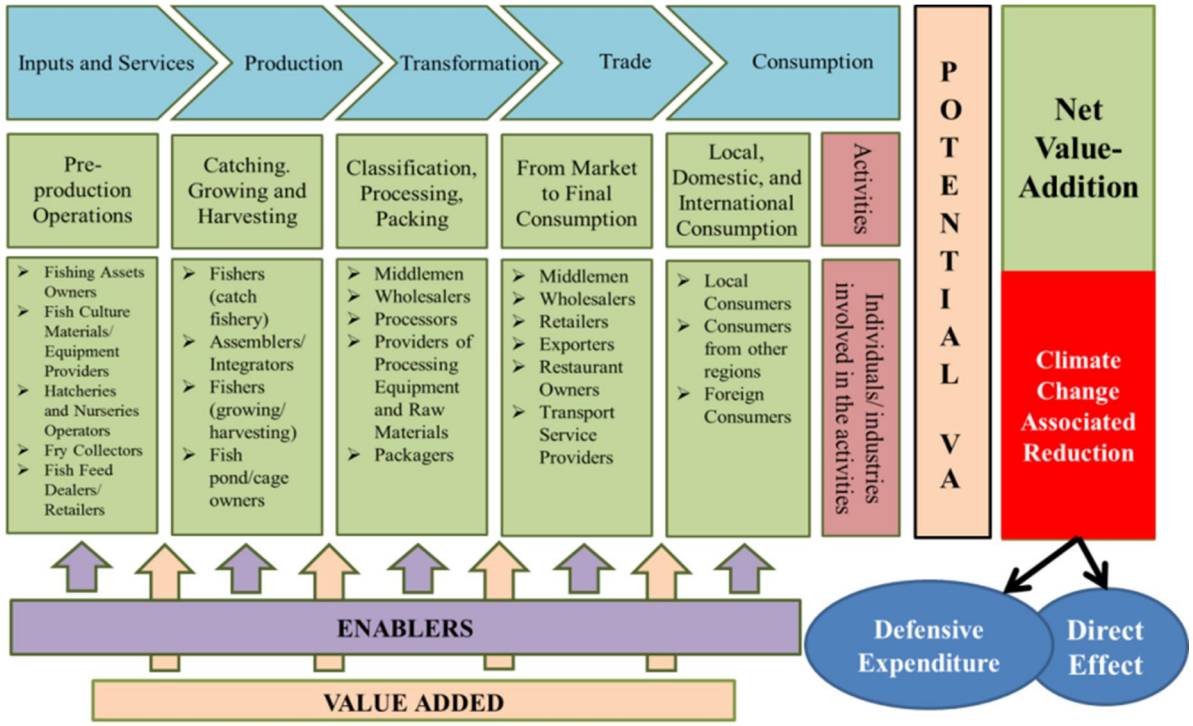

Fig. 1 Value Chain Analysis (VCA) framework. *Source: Ramirez et al. (2017)*

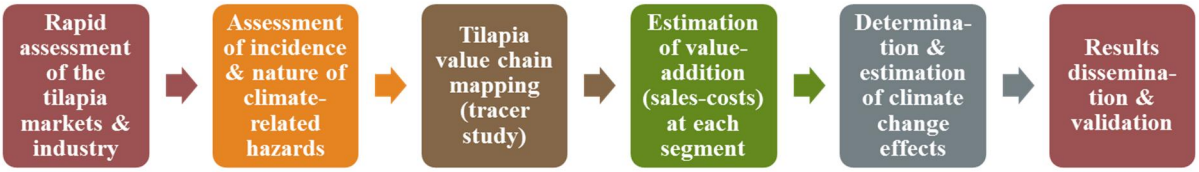

Fig. 2 Flow of Methodologies

3. Results and Discussion

A summary of the tilapia value chain in Luzon is shown in Fig. 3. Hatchery and nursery farms supply fry or fingerlings to tilapia grow-out operators. An average of six months is allotted to grow tilapia before it can be harvested. Then, tilapia is made available for consumers in the market by various traders, such as broker-trader, harvester, assembler-wholesaler, and retailer.

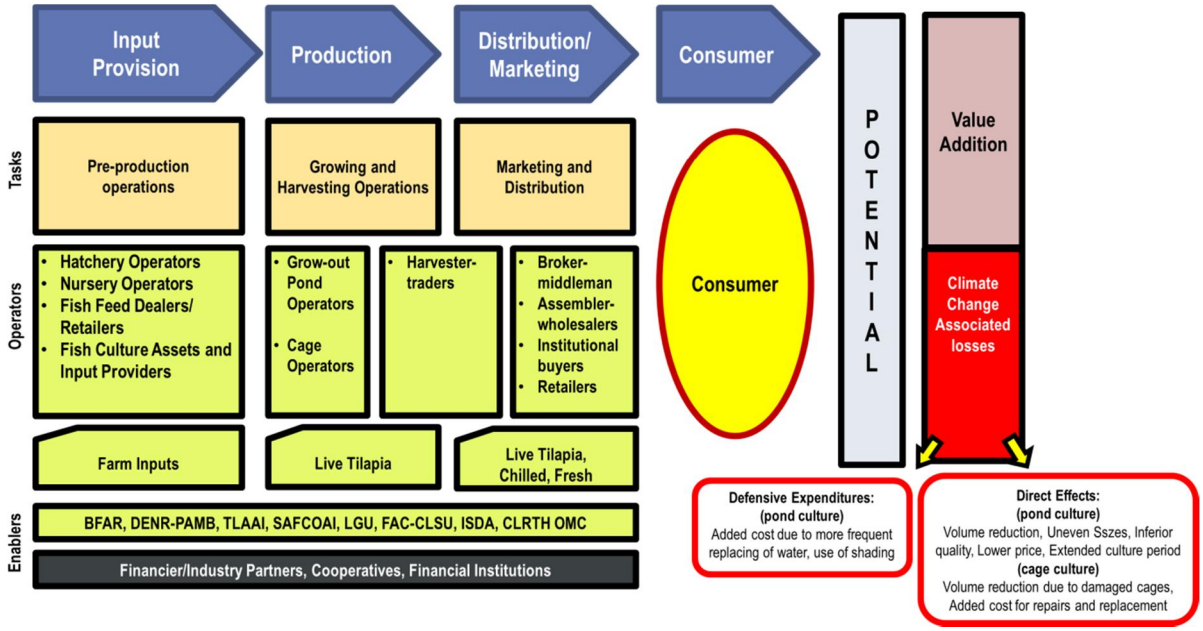

Fig. 3 Tilapia value chain map in Luzon.

Source: Ramirez et al. (2017)

The value chain in the Calabarzon region captures the movement of fresh tilapia coming from cage culture. Fish cage operators in Taal Lake obtain their fingerlings from nursery operators in Batangas, while nursery operators get tilapia fry from hatcheries in Laguna. At the end market, tilapia is sold either in live or chilled form. The harvesters of tilapia serve as the link between the cage operators and traders. From the farm, tilapia is often marketed within Batangas and nearby provinces in Southern Luzon. During the validation activity, participants indicated that tilapia from cages even reach areas in the Visayas. The primary climate change hazard (CCH) identified that the impacted chain involves Typhoon Glenda in 2014. All the players in value chain were adversely affected by the super typhoon. The product flow of tilapia produced in cages in the Calabarzon region is also illustrated (Fig. 4).

Using cost and returns analysis, the contribution to the value addition of all the players in the chain was computed, under the "without" and "with" CCH scenarios. Results indicated that cage operators interviewed had the greatest contribution of 41 percent in the value of tilapia along the chain under favorable weather conditions (Fig. 5, left panel), followed by the various types of traders. In the presence of extreme weather events (Fig. 5, right panel), revenues of cage operators dropped due to reduced volume in tilapia produced or diminished value/price at times of excessive supply/harvest of smaller sized tilapia. Meanwhile, costs increased due to faster depreciation of assets or additional cost for replacement or repair. Though the shares of the traders seem to be higher relative to other players, their actual value added declined under

the “with” CCH scenario. This is evidenced by the smaller-sized value chain “pie” indicating that the over-all benefits to the major value chain player dropped.

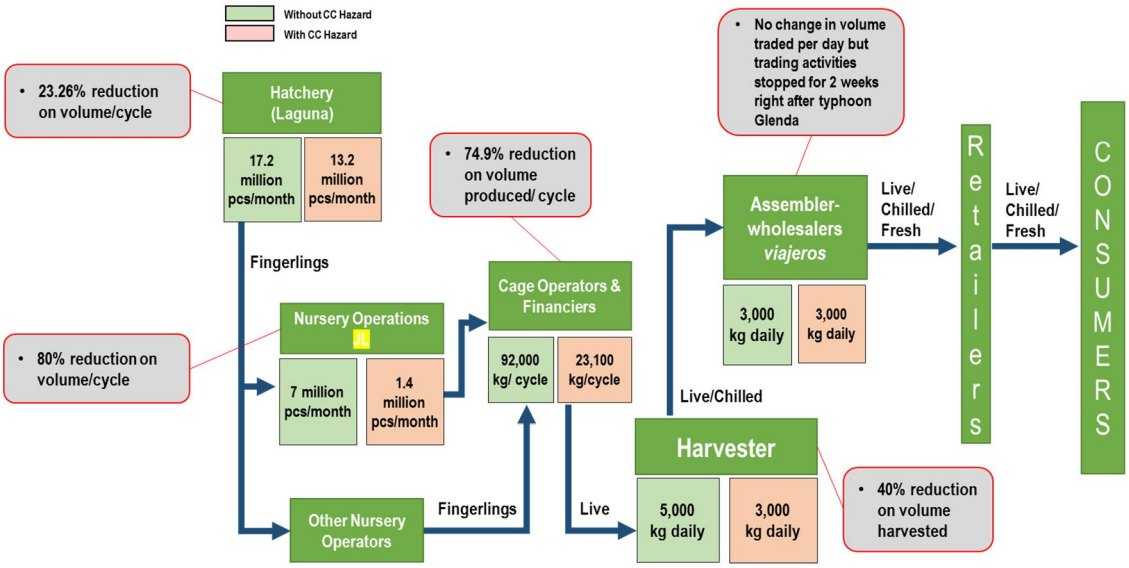

Fig. 4 Tilapia production flow in Calabarzon and effects of typhoon Glenda.

Source: Ramirez et al. (2017)

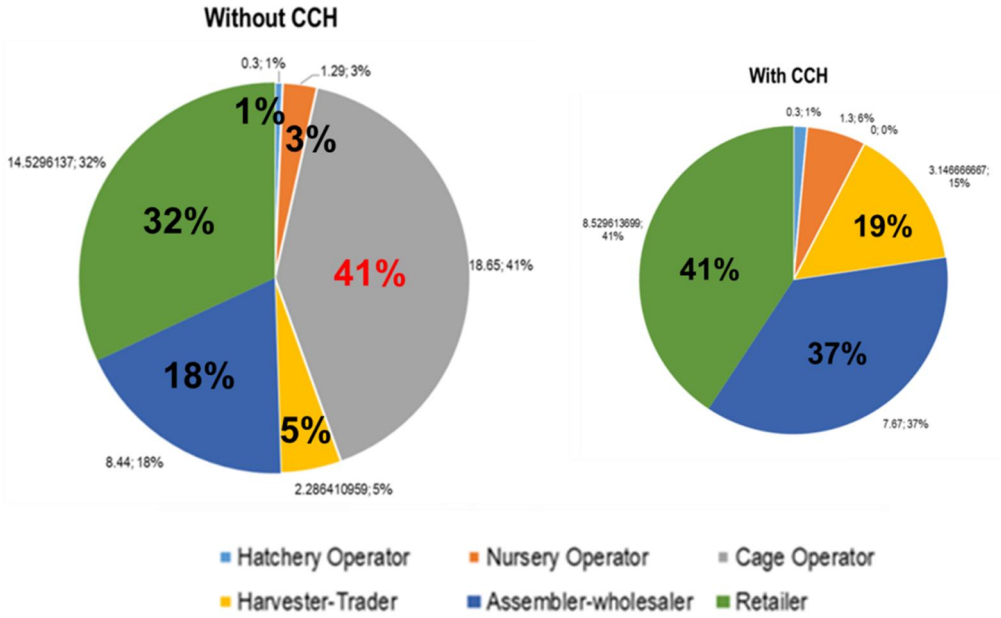

Fig. 5. Value addition shares of value chain players (without CCH; with CCH) in Calabarzon

Source: Ramirez et al. (2017)

4. Conclusions

The devastating effects of extreme weather events directly affected inputs and production subsystems and indirectly the downstream players in the tilapia value chains in Calabarzon. Adverse impacts to tilapia cage culture are mainly reflected via loss of harvest/value of harvest, and faster depreciation of aquaculture assets or repair/replacement cost. Impacts of climate-induced hazards lead to redistribution of value-added shares among players and a smaller-sized pie. The present study revealed that a comprehensive understanding of the industry/value chain is essential in identifying a holistic and more inclusive approach to address impacts of climate change. Most disaster-related programs in the Philippines tend to focus on individual/ household safety, emergency housing, relief goods and general infrastructure rehabilitation. While these may be on the top priority, appropriate attention must also be given to business-related resiliency programs. Lastly, there is the need for more pro-active (rather than reactive) mechanisms to support the capacities of value chain members in responding to these climate change related threats, such as vulnerability mapping, zoning/spatial planning, among others.

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A Combination of Fresh Feeds Enhances Sperm Quality of Domesticated Male Black Tiger Shrimp (*Penaeus monodon*)

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Sittikankeaw, K., Leelatanawit, R., Phuengwas, S., Khudet, J., Phomklad, S., Wongtriphop, S., Karoonuthaisiri, N., 2019. A combination of fresh feeds enhances sperm quality of domesticated male black tiger shrimp (*Penaeus monodon*), in: Proceeding of the Sixth International Symposoium on Cage Aquaculture in Asia. Klinbunga, S., Karoonuthaisiri, N., Piyapattanakorn, S. (Eds.). Asian Fisheries Society, pp. 35-40.

Abstract

Poor reproductive maturation of male captive black tiger shrimp is one of the major problems for shrimp industry. Fresh feeds were commonly used as maturation diet. This study examined effects of fresh feed combinations on sperm quality of male captive broodstock. Five shrimp groups were fed with different feeds: grow-out pellet, broodstock pellet, squids, polychaetes and a combination of polychaetes and squids. Growth rate, spermatophore weight, and total sperm count were measured after a 4-week feeding trial. All three fresh feed groups significantly improved growth rate, spermatophore weight and sperm count when compared to those of pellet groups. The analysis of nutrients and fatty acid profiles of the feeds is needed for future study to identify proper feed composition for successfully enhancing sperm performance and maturation of male captive black tiger shrimp.

Keywords: Feed combination; Sperm performance; Black tiger shrimp; *Penaeus monodon*

1. Introduction

The black tiger shrimp (*Penaeus monodon*) industry has been declining due to many farming problems. Poor reproductive maturation in captivity is one of the key problems preventing successful and sustainable domestication of this species. Natural live feeds (e.g., polychaetes, squid, mussels, crab, and artemia) have been used to enhance reproductive maturation (Rothlisberg, 1998; Peixoto et al., 2005) due to their high levels of polyunsaturated fatty acids (PUFA), which were reported to be involved in reproductive functions in female (Coman et al., 2007; Hoa et al., 2009; Coman et al., 2011) and male penaeid shrimp (Meunpol et al., 2005; Braga et al., 2010; Perez-Velazquez et al., 2003; Shailender et al., 2012).

We previously found that sand polychaetes (*Perinereis nuntia*) benefited growth rates, survival rate, and physical appearance as well as male reproductive maturation of the captive broodstock (Leelatanawit et al., 2014). However, in shrimp farming, maturation diets generally consist of combinations of live feeds or combinations of live feeds and commercial dry pellets

(Coutteau et al., 2001) to synergize the enhancing effects on reproductive maturation in various shrimp species. In the Pacific white shrimp (*Litopenaeus vannamei*), combination of marine bloodworms (*Glycera dibranchiata*) and squid at 1.5:1 ratio and other dietary treatments were used to study reproduction in male, and results showed that the live feed combination was not nutritionally optimal for male broodstock (Perez-Velazquez et al., 2003)

In *P. monodon,* only limited numbers of studies on feed effects were available. For instance, sexually mature shrimp was used as a supplement to substitute squid and bivalves in the maturation diets; however, it was not able to increase the reproductive performance for both female and male domesticated *P. monodon* (Coman et al., 2007). Meunpol et al., 2005 compared a combination of live feeds; sand and mud polychaetes (*Perinereis* spp. and *Marphysa* spp.), squid, and mussels and experimental pellets containing a highly unsaturated fatty acid (HUFA) ratio close to polychaetes for their ability to enhance reproductive performance of the domesticated male *P. monodon*. Nutritional and HUFA contents of each live feed and other feeds were also analyzed, but not for live feed combination (Meunpol et al., 2005). Moreover, a recent study has compared the effect of live feeds including polychaetes, squid and oyster on male reproductive maturation of wild *P. monodon* (Shailender et al., 2012). However, effects of live feeds (polychaetes and squid) and their combinations to sperm performance of the male domesticated *P. monodon* have never been studied before.

Therefore, this study aims to examine effects of fresh feeds and their combination on reproductive maturation of male broodstock in captivity. The spermatophores from each feed group before and after a 4-week feeding trial were assessed.

2. Materials and Methods

2.1 Experimental broodstocks

Domesticated male and female shrimp (11-month-old) were cultured at Shrimp Genetic Improvement Center (SGIC), Suratthani, Thailand.

2.2 Feed preparation

Feeds used in this experiment were commercial grown-up (for juvenile growing up to adult marine shrimp) and broodstock (for marine shrimp broodstock) pellets (Charoen Pokphand Foods PCL.; CPF, Thailand). Nutrient values for grown-up pellet reported by the company were > 35% proteins, > 5% lipids, < 12% moisture, and < 4% fibers; and those for broodstock pellet were > 50% proteins, > 8% lipids, < 12% moisture, and < 4% fibers.

In addition, 6-month-old live sand polychaetes (*Perinereis nuntia*) cultured at SGIC, and frozen squid (Marine Leader, Thailand) were also included in the experiments. The conditions used to culture the polychaetes mimic natural habitat in the intertidal zone by transferring seawater out in the evening and adding new seawater in the subsequent morning. Sand polychaetes were fed 2 twice daily with another commercial feed (CFP, Hi-grade 5101; > 38% proteins, > 5% lipids, < 11% moisture, and < 3% fibers).

2.3 Feed experiments and sample collection

This experiment was carried out from January 2016 to February 2016 at SGIC, Suratthani, Thailand. A total 260 domesticated male shrimp were randomly separated into 5 ponds (3 x 5 x 0.05 m^3, 52 shrimp/pond), acclimatized for one week prior to the experiment, and fed with the commercial grown-up pellet. Five experimental groups were shrimp fed with (1) commercial grown-up pellet (control group), (2) commercial broodstock pellet, (3) polychaetes, (4) squid, and (5) combination of polychaetes and squid (ratio 1:1). Each group was fed 5 times daily (8:00, 11:00, 14:00, 16:30, and 22:00 h) with 2% (commercial pellets;

groups 1-2) and 7% feed/shrimp weight/day (fresh feeds; groups 3-5) for four weeks. Percentage of feed was determined upon shrimp consumption as previously reported (Millamena and Quinitio, 2000).

The experiment was conducted at 29.3 ± 0.7 ppt seawater which was circulated daily (50% rate) and continuous aeration to maintain the dissolved oxygen level at above 5 mg/l. Water quality (temperature, pH and toal ammonia-N) during the experiment period were controlled and they were 27.6 ± 1.3°C, 8.14 ± 0.12 and 0.44 ± 0.29 mg/l, respectively. The spermatophore samples were randomly collected from the fifth pereiopod of shrimp from each group before feeding (control: Week 0; $N = 20$ per feeding group) and after feeding 4 weeks (test: Weeks 4; $N = 20$ per feeding group) for measurement of bodyweight and spermatophore weight.

2.4 Sperm performance assessment

Sperm weight and sperm count were assessed as the following. For each feeding group, 10 shrimp per time point were randomly selected for sperm count. Both spermatophores were collected and separately homogenized in a calcium-free sea water solution to prevent variation in sperm morphology. After debris sediment, sperms were counted using a hemacytometer as previously described (Leung-Trujillo and Lawrence, 1987) under a light microscopy (Olympus CH30). The counting of total sperm number was repeated with 8 aliquots from a sample (4 aliquots/ 1 side of spermatophore; 2 spermatophore/sample). Each aliquot was counted 5 times when sperm number is greater than 100. If sperm number is less than 100, all 25 holes of the middle hemacytometer were counted. The average counts of all aliquots were used to represent the sperm counts for that shrimp. For each group at each time point, the average counts from all the shrimp were used.

2.5 Data analysis

The body weight of shrimp in different groups at the beginning (week 0) and at the end (week 4) of a feeding trial were statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple range test. To determine effects of each feed formula on the growth, the body weight of experimental shrimp begin and after fed with the same feed was analyzed using an independent t-test. To compare effects of different feed formulas on sperm weight and total sperm number, data were analyzed with ANOVA and a *post hoc* test described previously. Significant differences were considered if $P < 0.05$.

3. Results and Discussion

3.1 Effects of fresh feeds on growth of male domesticated brooders

Body weight was recorded as an indicator for growth of the shrimp (Table 1). Body weights of shrimp in all groups before feeding (Week 0) were not different ($P > 0.05$), whereas body weights of shrimp fed with fresh feeds (polychaetes, squid, and combination of polychaetes and squid) were significantly higher than those fed with grown-up and broodstock pellets at Week 4 after feeding. When compared between Week 0 and Week 4 of the same feed, body weights of the polychaetes-fed and combination-fed groups were significantly higher than those at Week 0 (Table 1).

Table 1 Growth of shrimp fed with different feeds before (Week 0) and after a feeding trial (Week 4).

Feed groups	Weight (g)	
	Week 0	Week 4
Commercial pellet	49.07±5.18^{a,A}	46.54±4.00^{a,A}
Broodstock pellet	49.11±4.41^{a,A}	46.43±3.60^{a,A}
Polychaetes	45.23±4.48^{a,A}	51.71±4.05^{a,B}
Squid	47.97±4.62^{a,A}	51.30±4.66^{a,B}
Combination of polychaetes and squid	48.06±3.39^{a,A}	51.78±3.69^{a,B}

Different small letters indicate statistical differences among the different feeds at the same time point ($P < 0.05$). Capital superscripts indicate a statistical differences between two time points for the same feed group ($P < 0.05$).

The growth benefits from polychaetes observed in this study agree with our previous report (Leelatanawit et al., 2014). The results from this study further demonstrate that beside polychaetes, squid and fresh feed combination also provided equal benefits to shrimp growth but more cost-effective than the use of polychaetes alone.

3.2 Effects of fresh feeds on reproductive performance of male domesticated P. monodon

The relative increases in spermatophore weight and sperm number of polychaete and combination groups were significantly higher than other groups (Fig. 1). This result agreed with our previous work that polychaetes helped to increase total number of sperms (Leelatanawit et al., 2014).

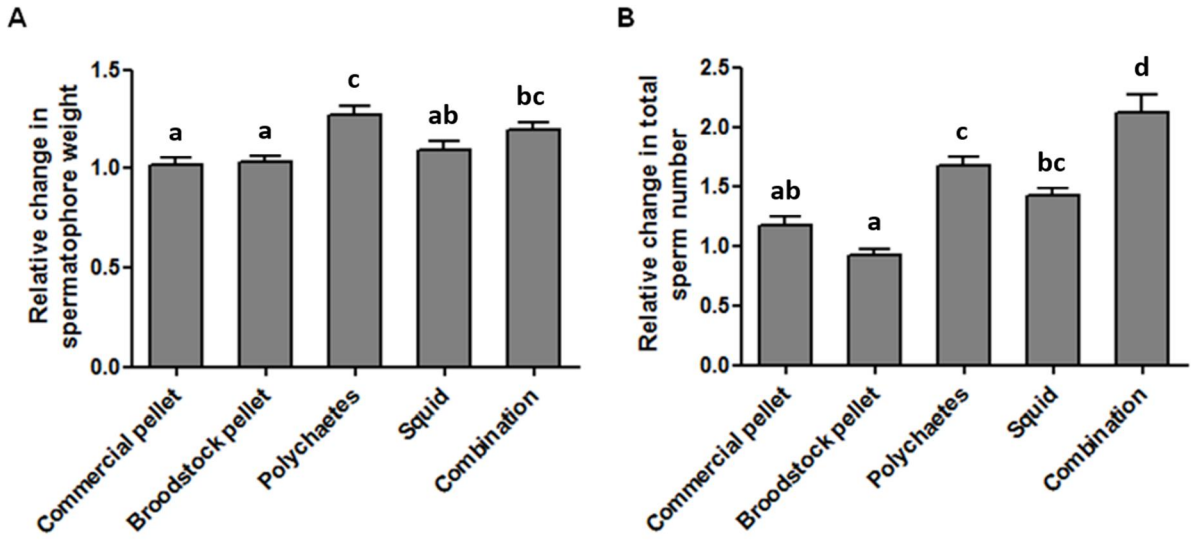

Fig. 1 Relative change in spermatophore weight (A) and in total sperm number (B). Relative change was calculated from the ratio between the value of shrimp after (Week 4) to before (Week 0) a feeding trial. Error bars indicate a standard derivation (SD) value. Different letters above the bars indicate statistical difference among different feeds ($P < 0.05$).

In conclusion, the fresh feed combination gives a better result in term of sperm number comparing with polychaetes or squid alone. However, the ratio 1:1 might not be optimal for increasing sperm performance of the captive male shrimp. Our results suggest that a suitable ratio between polychaetes and squid needs to be examined to further improve sperm quality of male captive black tiger shrimp.

Acknowledgments

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Single Nucleotide Polymorphism (SNP) in *Molt-Inhibiting Hormone* Gene and Its Association with Growth Parameters of the Black Tiger Shrimp *Penaeus monodon*

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Rongmung, P., Menasveta, P., Klinbunga, S., Khamnamtong, B., 2019. Single nucleotide polymorphism (SNP) in *Molt-inhibiting hormone* gene and its association with growth parameters of the black tiger shrimp *Penaeus monodon*, in: Proceeding of the Sixth International Symposoium on Cage Aquaculture in Asia. Klinbunga, S., Karoonuthaisiri, N., Piyapattanakorn, S. (Eds.). Asian Fisheries Society, pp. 41-49.

Abstract

Molecular markers linked to commercially important phenotypes are useful and can be applied for selection of juveniles and broodstock with desired traits in breeding programs of economically important species. In this study, nucleotide sequences of *molt-inhibiting hormone* of black tiger shrimp, *Penaeus monodon* (*PmMIH*) from five families of the fifth generation (G5) of domesticated *P. monodon* were sequenced and multiple aligned. Several SNPs were found. Sequence analysis using the NEBCutter program suggested that a G>T_{217} SNP can be simply analyzed by restriction analysis with *Bst*API. As a result, association analysis between *PmMIH* genotypes and the body weight of a domesticated G7 stock (four full-sib families of 5-month-old juveniles cultured in the same concrete pond) was investigated. Juveniles exhibiting a G/T_{217} genotype (15.52 ± 0.42 g, N = 161) had a significant greater average body weight than those exhibiting a G/G_{217} genotype (13.73 ± 0.74, N = 82). Subsequently, additional specimens from 19 full-sib families of G7 juveniles cultured in 5 different concrete ponds (4, 4, 4, 4 and 3 families/pond, N = 60 for each pond) were further examined. Results were consistent when the data were combined to cover all examined samples (18.11 ± 0.63 g for G/T_{217} juveniles and 15.90 ± 0.77 g for G/G_{217} juveniles; N = 314 and 159, respectively). Our results indicate the potential of a G>T_{217} SNP of *PmMIH* for differentiation of fast from slow growing juveniles in the selective breeding program of *P. monodon*.

Keywords: Black tiger shrimp; *Penaeus monodon*; MIH, Growth; SNP; PCR-RFLP

1. Introduction

The black tiger shrimp (*Penaeus monodon*) is one of the economically important species in Thailand (Withyachumnarnkul et al., 1998). Genetic improvement programs for increasing productivity of this species are required to meet the increasing demands of the industry (Argue et al., 2002; Benzie, 1997 and 1998; Cock et al., 2009 and 2010; Goyard et al., 2001 and 2002).

Genetic markers linked with growth or those regulating growth mechanisms can be directly applied to improve artificial selection processes of this economically important species (Jung et al., 2011 and 2013).

Single nucleotide polymorphisms (SNPs) are one base changes including substitutions, insertion or deletion (indels) occurring in the same genomic position of the DNA segments of different individuals. Informative SNP may linked with commercially important phenotypes (e.g. growth or disease resistance). SNP analysis is one of the efficient approaches for discovery of genes exhibiting major contribution in production traits of commercially important species (He et al., 2011; Liu and Cordes, 2004; Tao and Boulding, 2003). SNPs are especially important if they cause differences in economic traits, or the mutations are linked to the phenotypes of interest. Identification of functionally important growth genes in agricultural and aquaculture species can be used to increase the accuracy of selection for desired traits, thereby increasing the production efficiency.

In crustacean, *crustacean hyperglycemic hormone* (*CHH*) has been investigated in several decapods species. *CHH* plays major roles in carbohydrate and lipid metabolism, and also influences molting, reproduction, and osmoregulatory functions. Thanh et al. (2010) screened SNPs in *actin* and *CHH* genes and evaluated correlation between SNPs with individual growth performance in the giant freshwater prawn, *Macrobrachium rosenbergii*. Four SNPs in the *actin* gene (single SNP in the AC2 fragment and three SNPs in the AC3 fragment) and ten SNPs in the *CHH* gene (single SNP in the 5´UTR and nine SNPs in the intronic region) of 243 individuals of offspring were found. No association with growth traits in the *actin* gene was observed in the examined sample, while four intronic SNPs of *CHH* exhibited highly significant associations with individuals growth performance (body weight, carapace length and standard length) ($P < 0.05$). Of these, homozygous individuals (GG) at CH3 g.2402 had a significantly slower growth rate than heterozygous individuals (GT). In contrast, individuals with the AA genotype at CH3 g.2561 had a significantly faster growth rate than those with the GA genotype. Homozygotes at CH3 g.2407 (GG) and g.2409 (AA) showed faster growth rate than heterozygotes (GA and GA); however, the significant effect was only on the body weight. A further haplotype-trait association analysis confirmed that these four SNP markers were in linkage disequilibrium, and the specific haplotype TGAA had significant associations with high growth ($P < 0.01$). The findings allow increasing the efficiency of the selection process in *M. rosenbergii*.

Glenn et al. (2005) examined association analysis of SNP of *alpha-amylase* (*AMY2*) and *cathepsin-L* (*CTSL*) and the body weight in 2 populations of *Litopenaeus vannamei* (LV1 and LV2, $N = 75$ and 30 with the mean body weight of 0.35 ± 0.06 and 2.52 ± 0.30 g, respectively) and a mapping population of *P. monodon* ($N = 41$ but the body weight of examined shrimp was not available) of investigated shrimp. SNP genotypes were carried out using PCR-RFLP of *AMY2* with *Sca I* and *CTSL* with *Pvu* II. Neither polymorphism of *AMY2* nor *CTSL* was found to be significantly associated with the mean body weight of LV1 and LV2 populations ($P > 0.05$).

Molt-inhibiting hormone (MIH) is involved in the regulation of the length of the intermolt period and proposed to be one of the important neuropeptides controlling growth of shrimp. Yu et al. (2006) examined polymorphism in the amplified *MIH* gene segments in a full-sib mapping population ($N = 41$) of *P. monodon* developed by the Australian Institute of Marine Science and the Commonwealth Scientific and Industrial Research Organization. SNPs were found in the *MIH* gene segments but all the 41 full-sib individuals examined were

heterozygous. Neither association analysis nor linkage mapping could be analyzed in this sample set.

In the present study, polymorphism of *PmMIH* in five full-sib families of the fifth generation (G5) of domesticated *P. monodon* was initially analyzed by DNA cloning and sequencing. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was applied for detection in the subsequent generation of *P. monodon*. Relationships between genotypes of *PmMIH* and body weight of the G7 of 5-month-old juveniles were examined. The information implied a possible application of a SNP in *PmMIH* for selection of fast-growing *P. monodon*.

2. Materials and Methods

2.1 Experimental animals

Five full-sib families (Dam3, Dam48, Dam58, Dam103 and Dam111) of domesticated G5 generation of *P. monodon* cultured at Shrimp Genetic Improvement Center (SGIC, Suratthani, Thailand) were established. In addition, twenty-three full-sib families of the seventh generation (G7) of domesticated *P. monodon* were produced. Due to a limited number of available ponds, postlarvae 15 (PL15) of 4 families were cultured together in a single pond (25 pieces/ m^2; 25 m^2 concrete ponds) for 5 months. The body weight of each 5-month-old juvenile was measured. Pleopods were dissected out from each shrimp and kept at -20 °C until used.

2.2 Genomic DNA extraction

Genomic DNA was extracted from a frozen pleopod of each shrimp using a GF-1 Tissue DNA Extraction Kit (Vivantis). The quantity of extracted genomic DNA was spectrophotometrically estimated. DNA was kept at 4°C until further use.

2.3 Analysis of SNPs in PmMIH by PCR-cloning and sequencing

The *PmMIH* gene segment of each *P. monodon* individual was amplified, electrophoretically analyzed and eluted from the gel. The resulting product was cloned into pGEM-T Easy vector. One-tenth volume of each ligation reaction was transformed to *E. coli* JM109. Recombinant clones were selected by a *lacZ* system following standard protocols (Sambrook and Russell, 2001). Plasmid DNA was extracted. Nucleotide sequence of each insert was examined for both directions and searched against previously deposited data in GenBank using BlastN (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov). Nucleotide sequences of *PmMIH* of examined shrimp were multiple-aligned using Clustal W (Thompson et al., 1994).

2.4 PCR-RFLP analysis of a G/T_{217} SNP of PmMIH

Bioinformatic analysis was applied for searching restriction enzyme cutting sites within the *PmMIH* gene segment using the NEBCutter program. Genotypes of a G>T_{217} SNP were analyzed by restriction analysis with *Bst*API. Briefly, the amplified *PmMIH* gene segment of each *P. monodon* individual was amplified. Five microliters of each amplification reaction was electrophoresed through a 1.0% agarose gel to determine whether the resulting fragment was successfully amplified. Eight microliters of the amplification product were singly digested with *Bst*API using the conditions recommended by the manufacturer. The digests were electrophoretically analyzed through 2.0% agarose gels and visualized after ethidium bromide staining (Sambrook and Russell, 2001).

2.5 Data analysis

Statistical analysis was carried out to determine whether shrimp carrying different genotypes of a G>T_{217} SNP possessed significantly different body weight using an independent t-test (for bi-allelic SNP with two genotypes found in this study). Results were considered to be significantly different if $P < 0.05$.

3. Results

The amplified product of 461 bp in size was obtained from amplification of *PmMIH* against genomic DNA of 5-month-old *P. monodon* juveniles (Fig.1). Nucleotide sequences of *PmMIH* from five families of domesticated *P. monodon* were cloned and sequenced. The amplified *PmMIH* gene segment contained an intron at positions 207-442. Five SNPs (positions 31, 217, 263, 314 and 391) were found from multiple alignments of nucleotide sequences of *PmMIH* in the G5 sample of *P. monodon*. Of these, a SNP at position 31 of the amplified gene segment was located in the exon while four SNP positions (213, 217, 314 and 314) were located in the intron region.

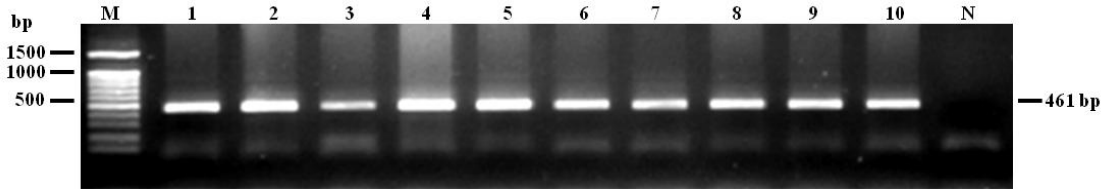

Fig. 1 A 1.5% ethidium bromide-stained agarose gel showing the amplification product of the *PmMIH* gene segment against genomic DNA of different individuals of 3-month-old *P. monodon* (SNP3A, Lanes 1-10). Lanes M and N are a 100 bp DNA marker and the negative control (without genomic DNA template), respectively.

```
MIH-Dam3-03      TCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTAATATT 240
MIH-Dam3-04      TCTAAGGATTTATACATATAGAGACAGGGCGTAGATTGCATATGGGTACGAAGTAATATT 238
MIH-Dam3-93      TCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTGATATT 240
MIH-Dam3-94      TCTAAGGATTTATACATATAGAGACAGGGCATAGATGGCATATGGGTACGAAGTAATATT 240
MIH-Dam48-03     TCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTAATATT 240
MIH-Dam48-05     CCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTAATATT 240
MIH-Dam48-101    TCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTAATATT 240
MIH-Dam48-102    TCTAAGGATTTATACATATAGAGACGGGGCATAGGTGGCATATGGGTACGAAGTAATATT 240
MIH-Dam58-01     TCTAAGGATTTATACATATAGAGACAGGGCATAGATGGCATATGGGTACGAAGTAATGTT 240
MIH-Dam58-04     TCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTAATATT 240
MIH-Dam58-103    TCTAAGGATTTATACATATAGAGACAGGGCATAGATGGCATATGGGTACGAAGTAATATT 240
MIH-Dam58-105    TCTAAGGATTTATACATATAGAGACAGGGCATAGATGGCATATGGGTACGAAGTAATATT 240
MIH-Dam103-01    TCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTAATATT 240
MIH-Dam103-02    TCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTAATATT 240
MIH-Dam103-103   TCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTAATATT 240
MIH-Dam103-104   TCTAAGGATTTATACATATAGAGACAGGGCATAGATTGCATATGGGTACGAAGTAATATT 240
MIH-Dam111-02    TCTAAGGATTTATACATATAGAGACAGGGCATAGATGGCATATGGGTACGAAGTAATATT 240
MIH-Dam111-05    TCTAAGGATTTATACATATAGAGACAGGGCATAGATGGCATATGGGTACGAAGTAATATT 240
MIH-Dam111-104   TCTAAGGATTTATACATATAGAGACAGGGCATAGATGGCATATGGGTACGAAGTAATATT 240
MIH-Dam111-105   TCTAAGGATTTATACATATAGAGACAGGGCATAGATGGCATATGGGTACGAAGTAATATT 240
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Fig. 2 Example of aligned nucleotide sequence of *PmMIH* in five families of the G5 sample of domesticated *P. monodon*. A G>T_{217} SNP which was able to detect by PCR-RFLP analysis is highlighted. Polymorphism found in only one examined individual was considered as sequence variation.

Sequence analysis using the NEBCutter program suggested that the SNP at the position 217 (G>T_{217}) can be detected by restriction analysis with *Bst*API. PCR-RFLP was then developed (Fig. 3) and further applied for analysis of association between a G>T_{217} SNP and growth parameters of domesticated G7 samples of *P. monodon*.

Results from PCR-RFLP against genomic DNA of the 4S7 sample (G7, $N = 243$) indicated that two genotypes; G/G_{217} (461 bp) and T/G_{217} (461, 246 and 215 bp) were found as predicted from nucleotide sequences (Fig. 3). However, a T/T_{217} (246 and 215 bp) was not observed. Association between genotypes of *PmMIH* and growth parameters of *P. monodon* juvenile in cultured ponds was statistically analyzed. Results indicated that 5-month-old juveniles exhibiting a G/T_{217} genotype (15.52 ± 0.42 g, $N = 161$) had a significant greater average body weight than those exhibiting a G/G_{217} genotype (13.73 ± 0.74, $N = 82$).

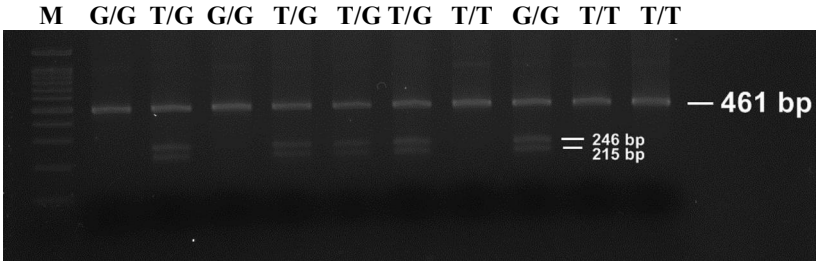

Fig. 3 PCR-RFLP analysis of different individuals of *P. monodon* juveniles exhibiting G/G_{217} (461 bp) and G/T_{217} (461, 246, 215 bp) genotypes of *PmMIH*. Lane M is a 100 bp DNA marker.

In addition, specimens from additional 19 full-sib families of G7 juveniles cultured in 5 different concrete ponds (4, 4, 4, 4 and 3 families/pond, $N = 60$ for each pond) were further analyzed. Although results did not reveal statistical significant differences between shrimp having different genotypes of a G>T_{217} SNP in small sample sizes of the G7 samples, a trend of differences in growth parameters of juveniles with different genotypes (considered as practical differences in this study) was observed. In the 1S7 sample, juveniles with a G/G_{217} genotype seemed to have a greater average body weight and total length than those with a G/T_{217} genotype. The results were in the opposite direction in 2S7, 3S7, 4S7, 5S7 and 6S7 samples where those with a G/G_{217} genotype had lower average body weight and total length than those with a G/T_{217} genotype. When all examined samples were pooled, juveniles exhibiting a G/T_{217} genotype (18.11 ± 0.63 g; $N = 314$) showed a significant greater average body weight and total length than those exhibiting a G/G_{217} genotype (15.90 ± 0.77 g; $N = 159$ $P < 0.05$; Table 1).

4. Discussion

The primary goal of genetic selection is the improvement of growth performance of aquaculture species. Understanding association between genotypic and phenotypic causes of variation of growth in commercially cultivated species is a main interest of the aquaculture industry (Johnston, 1999; Kang et al., 2002; Liu et al., 2012). Presently, information on correlation between genotypic and phenotypic variations in penaeid shrimp is rather limited. Identification of molecular markers linked with growth or those regulating the growth mechanisms can be applied to improve artificial selection processes of *P. monodon*. Although the genes that affect a polygenetic trait such as growth can typically be identified through the genetic linkage maps, potential candidate genes can also be selected based on a known contribution between physiological or biochemical functions of a particular trait (Tao and

Boulding, 2003). A number of putative candidate genes related to growth and muscle development have been identified in several economically important species (Dekkers, 2004; De-Santis et al., 2007; Zhang et al., 2009). These candidate genes are promising for the future development of gene-assisted selection (GAS) markers (De-Santis and Jerry, 2007; Jung et al., 2013; Liu and Cordes, 2004; Tao and Boulding, 2003).

Table 1 Relationships between genotypes of *PmMIH* and growth parameters of 5-month-old juveniles of *P. monodon*

Sample/Pattern	*N*	Average body weight ± SEM (g)
1S7 (4 families)		
G/T_{217}	34	17.90±2.41[a]
G/G_{217}	25	19.11±2.54[a]
2S7 (4 families)		
G/T_{217}	38	16.75±1.69[a]
G/G_{217}	18	14.07±2.34[a]
3S7 (4 families)		
G/T_{217}	30	22.01±2.50[a]
G/G_{217}	30	18.12±1.99[a]
4S7 (4 families)		
G/T_{217}	161	15.52±0.42[b]
G/G_{217}	82	13.73±0.74[a]
5S7 (4 families)		
G/T_{217}	35	19.75±2.23[a]
G/G_{217}	25	14.97±3.11[a]
6S7 (3 families)		
G/T_{217}	35	24.09±2.17[a]
G/G_{217}	25	17.70±3.12[a]
Pooled 1S7-6S7 (23 families)		
G/T_{217}	314	18.11±0.63[b]
G/G_{217}	159	15.90±0.77[a]

The same superscripts indicate non-significant differences between shrimp carrying different genotypes ($P > 0.05$).

Molecular mechanisms involving growth of penaeid shrimp have long been of interest by aquaculture industries (Jung et al., 2013). Previously, a genetic linkage map of a full-sib F_2 intercross family of *Marsupenaeus japonicus* was constructed using AFLP analysis. A homologue of gene encoding the elongation of very long chain fatty acids-like (ELOVL) protein family resided within the QTL peak was characterized. However, association between SNP and growth rates or expression levels of the *ELOVL* gene was not examined (Lyons et al., 2007).

In *P. monodon*, Prasertlux, et al. (2010) reported the correlation between SNP in *RuvBL2* and growth rate of *P. monodon*. *ATP-dependent DNA helicase* (*RuvBL2*) is essential for growth in eukaryotes (Makino et al., 1999; Qiu et al., 1998). The full-length cDNA of *P. monodon RuvBL2* was 3791 bp and contained a 1392 bp open reading frame (ORF) corresponding to a polypeptide of 463 amino acids. Polymorphism of the amplified *PmRuvBL2* gene segment (484 bp containing an intron of 259 bp) was examined in commercially cultivated shrimp from the same pond (approximately 3 months old, average BW = 17.39 ± 4.36 g, $N = 359$) by single-strand conformational polymorphism (SSCP) analysis. Results indicated that the *P. monodon* with genotypes A (average BW = 19.277 ± 3.640 g, $N = 37$) and B (average BW = 19.293 ±

4.548 g, $N = 79$) was significantly greater body weight than those genotype C (average BW = 16.528 ± 3.847 g, $N = 93$) and D (average BW = 16.365 ± 4.378 g, $N = 124$). One exonic (G-A81) and two intronic (A-T196 and G-T248) SNPs corresponding to ATG, A[T/A]G, GAG and GAT for respective SSCP genotypes were found. Nevertheless, the identified SNP was not polymorphic when additional sample sets were examined. Therefore, additional molecular markers need to be determined.

In the present study, an additional SNP marker in *PmMIH* showing significant association with growth traits when preliminary tested in a limited number of domesticated *P. monodon*. Its potential for further applications was observed. Association between a candidate SNP marker ($G>T_{217}$) and body weight of *P. monodon* was examined in a larger number of families. Two genotypes (G/T_{217} and G/G_{217}) were found in each of 1S7- 6S7 samples. Disregarding the 4S7 sample where a large sample size was examined, the analysis did not reveal significant growth parameters in juveniles carrying different SNP genotypes in each pond but a clear trend of phenotypic differences was observed. When data from 23 full-sib families cultured in six ponds were combined, G/T_{217} ($N = 314$) showed a greater average body weight and total length than those with G/G_{217} ($N = 159$). Results obtained confirm the potential of this SNP marker to be used for genetic improvement of domesticated *P. monodon*.

The genetic improvement and other biotechnological applications are crucial to the future sustainable development of *P. monodon* industry. PCR-RFLP was applied to examine the association between SNP genotypes of *PmMIH* and growth traits of *P. monodon* juveniles. The findings about relationships between genotypes and phenotypes (commercially important traits like growth parameters of *PmMIH*) can be applied to assist the ongoing domestication programs of this economically important species.

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Association between Single Nucleotide Polymorphisms (SNPs) of *X-box Binding Protein 1* and Growth of the Black Tiger Shrimp *Penaeus monodon*

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Abstract

Growth-related markers are useful and can be further applied for genetic improvement of economically important species. Here, single nucleotide polymorphism (SNP) in *X-box binding protein 1* (*PmXbp1*) of the black tiger shrimp (*Penaeus monodon*) was examined. Association between genotypes of *PmXbp1* and the average body weight and total length of a 3-month-old domesticated stock of *P. monodon* (SNP3A sample established from one female and two males; average body weight and total length = 12.32 ± 0.40 g and 11.30 ± 0.12 cm, N = 162) were tested using single-strand conformational polymorphism (SSCP) analysis. The average body weight and total length of juveniles carrying pattern A (corresponding to a T/T_{27} SNP; 15.07 ± 0.76 g and 12.14 ± 0.21 cm, N = 44) was significantly greater than that of juveniles carrying pattern B (corresponding to a T/C_{27} SNP; 11.37 ± 0.44 g and 10.99 ± 0.14 cm, N = 116) (P < 0.05). In addition, a SNP of *PmXbp1* in the 7th generation of domesticated *P. monodon* was investigated and a $G>A_{76}$ SNP was found by PCR-direct sequencing. A rapid technique for detection of this SNP was developed based on PCR amplification of specific alleles using real-time PCR (real-time PCR-based PASA). Association analysis was tested in four G7 families of 5-month-old juveniles reared in the same concrete pond. Results indicated that juveniles exhibiting a G/G_{76} genotype possessed significantly greater average body weight and total length (15.38 ± 0.40 g and 11.66 ± 0.12 cm, N = 204) than those carrying a G/A_{76} genotype (12.75 ± 1.16 g and 10.96 ± 0.31 cm, N = 32, P < 0.05).

Keywords: Black tiger shrimp; *Penaeus monodon*; Growth; SSCP; SNP

1. Introduction

The black tiger shrimp (*Penaeus monodon*) is one of the most economically important cultured species (Rosenberry, 2003). Breeding of pond-reared *P. monodon* is extremely difficult and rarely produced enough quality larvae desired by the industry resulting in overexploitation of the natural populations of *P. monodon* in Thai waters (Benzie, 1997 and

1998; Klinbunga et al., 2001; Laubier and Laubier, 1993). This leads to the significant reduction of the aquaculture production in Thailand since 2004.

However, *P. monodon* is an indigenous species with high value for the premium sized market. The domestication and selective breeding programs of this species would provide more reliable supply of seed stock and improve its production efficiency (Clifford and Preston, 2006; Coman et al., 2006; Khamnamtong et al., 2009; Makinouchi and Hirata, 1995). Rather than relying on wild-caught stocks, the shrimp industry will become more sustainable when using selectively bred stocks with improved culture performance, disease resistance and/or other commercially desired traits (Clifford and Preston, 2006). The success of genetic improvement program will help fulfilling the need for high-quality broodstock that can supply postlarval shrimps for the industry. In addition, the problems of overexploitation of wild *P. monodon* females will be subsided (Coman et al., 2006; Kenway et al., 2006; Withyachumnarnkul et al., 1998).

Molecular techniques, such as DNA-based technologies, have been developed to generate informative genetic maps of potential important traits of commercially cultured species (Liu and Cordes, 2004). Single nucleotide polymorphisms (SNPs) is a single base change in the DNA sequence, with three possible nucleotides at a given position. Analysis of gene-based SNPs is one of the efficient approaches for discovery of genes which significantly contribute in production traits of commercially important species (Liu and Cordes, 2004; Tao and Boulding, 2003).

The *X-box binding protein* gene encodes a bZIP-containing transcription factor that plays a key role in the unfolded protein response (UPR), an evolutionarily conserved signaling pathway activated by an overload of misfolded proteins in the endoplasmic reticulum (ER) (Hu et al., 2007; Yoshida et al., 2001). Recently, Souid et al. (2007) characterized *xbp-1* in *Drosophila*. *Dxbp-1* is ubiquitously transcribed. Loss of function of *Dxbp-1* induced a recessive larval lethality, thus, revealing an essential requirement for this gene.

In this study, the possible biological roles of *X-box binding protein 1* (*PmXbp1*) in growth of *P. monodon* was evaluated. Polymorphism of *PmXbp1* was examined using single-stranded conformational polymorphism (SSCP) and DNA sequencing. A SNP detection method was simplified to a polymorphic amplification of specific allele using real-time PCR (real-time PCR-based PASA) and applied for association analysis between SNP and growth parameters of *P. monodon* juveniles.

2. Materials and Methods

2.1. Experimental animals

A domesticated *P. monodon* (SNP3A) which was composed of two half-sib families of the 5th generation (G5) of *P. monodon* were established. Shrimp were cultured at the Broodstock Multiplication Center (BMC), Burapha University, Chanthaburi Campus and randomly collected after 3 months of cultivation (average body weight and total length = 11.83 ± 0.20 g and 11.23 ± 0.06 cm, respectively, N = 350). In addition, four full-sib family of the 7th generation (G7) of domesticated *P. monodon* were also established and cultured at Shrimp Genetic Improvement Center (SGIC), Surat Thani, Thailand. Juveniles were collected after 5 months of cultivation (average body weight and total length = 14.85 ± 0.32 g and 11.56 ± 0.10 cm, respectively, N = 300). Pleopods of each shrimp were dissected out and kept at -20°C until further required.

2.2. Genomic DNA extraction

Genomic DNA was extracted from a piece of the pleopod of *P. monodon* individuals using a phenol-chloroform-proteinase K method (Klinbunga et al., 2001). The concentration of extracted DNA was spectrophotometrically estimated. DNA was stored at 4 °C until used.

2.3. Single-strand conformational polymorphism (SSCP) analysis

The *PmXbp1* gene segment (F: 5′-TGATGAACTTCGGGACCTAA-3′ and R: 5′-CCTCAACGACAACTGCTGCG-3′) was amplified. SSCP was carried out against genomic DNA of 3-month-old *P. monodon* (SNP3A sample; average body weight and total length = 12.32 ± 0.40 g and 11.30 ± 0.12 cm, respectively, $N = 162$). The amplification product was analyzed by SSCP (Orita et al., 1989) using PROTEAN II xi cells (Bio-Rad). The PCR product (6 µL) was mixed with four volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol, and 10 mM NaOH), denatured in a boiling bath for 5 min, and immediately cooled on ice for 3 min. The denatured products of *PmXbp1* gene segment were electrophoretically analyzed (native 20% PAGE; 37.5:1 crosslink) at 250 V for 16 h at 4 °C. SSCP bands were visualized by silver staining.

2.4. Identification of SNP in the amplified PmXbp1 gene segment by cloning of the PmXbp1 gene segment and DNA sequencing

For the SNP3A sample, the *PmXbp1* gene segment of *P. monodon* juveniles representing each SSCP pattern ($N = 9$ each) was electrophoretically analyzed and eluted from the gel. The resulting product was cloned into a pGEM-T Easy vector and transformed to *Escherichia coli* JM109. Recombinant clones were selected by a lacZ system following standard protocols (Sambrook and Russell, 2001). The nucleotide sequence of each insert was examined for both directions and searched against previously deposited data in GenBank using BlastN (Altschul et al., 1990). Nucleotide sequences of *PmXbp1* of examined shrimp were multiple-aligned using ClustalW (Thompson et al., 1994).

In addition, SNPs in the amplified *PmXbp1* gene segment of representative individuals of 5-month-old 4S7 juveniles were also examined using the protocol described above.

2.5 Development of a SNP detection method using real-time PCR-based PASA

Genotying of a G>A_{76} SNP of each shrimp was carried out using a reverse primer (R: 5′-CCTCAACGACAACTGCTGCG-3′) and one forward allele-specific primer. (PmXbp1-F_G: 5′-AAGAAAATGCAGCACTGCA**G**-3' for allele G or PmXbp1-F_A: 5′-AAGAAAATGCAGCACTGCA**A**-3' for allele A). Accordingly, two reactions were performed for each individual. The target product was amplified in a 10 µl reaction volume containing 5 µl of 2x LightCycler 480 SYBR Green I Master (Roche), 100 ng genomic DNA template, 0.2 µM each primer. The amplification was carried out in a LightCycler 480 profiles using high stringent conditions or by touchdown profiles. After optimization, real-time PCR was carried out. Melting point analysis was performed by heating to 95°C for 15 s, cooled to 50°C for 15 s and reheated to 95°C for 15 s. Changes in fluorescence were measured during the final heating cycle and analyzed using LightCycler software. Each run contained the positive controls (specimens with known SNP of each gene) and a negative control (without genomic DNA template).

2.6 Data analysis

Statistical analysis was carried out to determine whether shrimp carrying different genotypes of a G>A_{76} SNP possessed significantly different body weight and/or total length using an independent t-test (for bi-allelic SNP with two genotypes found in this study). Results were considered significant if $P < 0.05$.

3. Results

3.1 Polymorphism and relationships between SSCP/SNP genotypes of PmXbp1 and the body weight of juvenile P. monodon

The amplified product of 186 bp in size was obtained from amplification of *PmXbp1* against genomic DNA of 3-month-old *P. monodon* juveniles. Three SSCP patterns were found in the examined SNP3A sample (Fig. 1). Of these, patterns A (N = 44) and B (N = 116) were collectively observed in 98.77% of examined specimens while pattern C was found in only two female individuals (1.23%). A single SNP ($T>C_{349}$ relative the full-length cDNA of *PmXbp1*; GenBank accession no. JN644920.1) was observed from cloning and sequencing of representative individuals exhibiting SSCP patterns A (T/T_{27}) and B (T/C_{27}).

Fig. 1 SSCP patterns of the *PmXbp1* gene segment amplified from genomic DNA of 3-month-old *P. monodon* (SNP3A).

Results clearly indicated that juvenile shrimp carrying SSCP genotype A (a T/T_{27} SNP) had a significantly greater average body weight and total length (15.07 ± 0.76 g and 12.14 ± 0.21 cm) than those carrying SSCP genotype B (a T/C_{27} SNP; 11.37 ± 0.44 g and 10.99 ± 0.14 cm, $P < 0.05$).

3.2 Polymorphism of PmXbp1 in 5-month-old juvenile of the G7 P. monodon sample

The amplified *PmXBP1*gene segment of 5-month-old specimens was cloned and sequenced (Fig. 2). One SNP was found at position 76 ($G>A_{76}$) of the amplified *PmXbp1* gene segment.

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PmXbp1-002  TGATGAACTTCGGGACCTAACAGCTGTTTTATCTGAACAGAACACTTGTCTTGCAGAAGA
PmXbp1-202  TGATGAACTTCGGGACCTAACAGCTGTTTTATCTGAACAGAACACTTGTCTTGCAGAAGA
PmXbp1-104  TGATGAACTTCGGGACCTAACAGCTGTTTTATCTGAACAGAACACTTGTCTTGCAGAAGA
            ************************************************************
PmXbp1-002  AAATGCAGCACTGAAGGAGATGTTGACAAAATGTACGTGTGGACAGGGCAGCACAGAGAG
PmXbp1-202  AAATGCAGCACTGAAAGAGATGTTGACAAAATGTACGTGTGGACAGGGCAGCACAGAGAG
PmXbp1-104  AAATGCAGCACTGAAGGAGATGTTGACAAAATGTACGTGTGGACAGGGCAGCACAGAGAG
            *************** ****************************************
PmXbp1-002  CAATGTCAACGAAGCCTTGAATGTCAGCTGTGACGACACGCACAACGCAGCAGTTGTCGT
PmXbp1-202  CAATGTCAACGAAGCCTTGAATGTCAGCTGTGACGACACGCACAACGCAGCAGTTGTCGT
PmXbp1-104  CAATGTCAACGAAGCCTTGAATGTCAGCTGTGACGACACGCACAACGCAGCAGTTGTCGT
            ************************************************************
PmXbp1-002  TGAGG
PmXbp1-202  TGAGG
PmXbp1-104  TGAGG
            *****
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Fig. 2 Nucleotide sequences of the amplified *PmXbp1* gene segment in different individuals of 5-month-old domesticated *P. monodon* (G7). A $G>A_{76}$ SNP is highlighted.

3.3 Development of real-time PCR-based PASA and association analysis between G>A$_{76}$ genotypes and growth parameters of juvenile P. monodon

The results from real-time PCR-based PASA clearly differentiated G/G and G/A genotypes found in the examined samples (N = 99). Only one amplified product was generated in shrimp with a G/G genotype by primers PmXbp1-F$_G$ + PmXbp1-R1 while PmXbp1-F$_A$ + PmXbp1-R1 primers did not generate the amplified product (Fig. 3). In contrast, two amplified products were generated from both primers in juveniles with a G/A genotype.

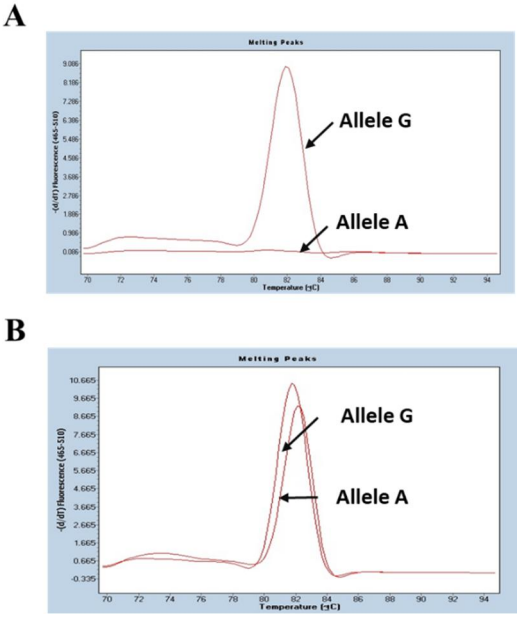

Fig. 3 Results from real-time PCR-based PASA for analysis of a G>A$_{76}$ SNP of *PmXbp1*. A single product was observed in juveniles representing a G>G$_{76}$ genotype (A) while two peaks were found in those representing a G/A$_{76}$ genotype (B).

Association analysis was performed against specimens from four G7 families of 5-month-old juveniles which were reared in the same concrete pond. Results indicated that juveniles exhibiting a G/G$_{76}$ genotype possessed significantly greater average body weight and total length (15.38 ± 0.40 g and 11.66 ± 0.12 cm, respectively, N = 204) than those carrying a G/A$_{76}$ genotype (12.75 ± 1.16 g and 10.96 ± 0.31 cm, respectively, N = 32, P < 0.05) (Table 1).

Table 1 Association analysis between different G>A$_{76}$ genotypes of *PmXbp1* and growth parameters of 5-month-old *P. monodon* (N = 266).

Genotype	*N*	Average body weight ± SEM (g)	Average total length ± SEM (cm)
G/G$_{76}$	204	15.38 ± 0.40^a	11.66 ± 0.12^b
G/A$_{76}$	32	12.75 ± 1.16^a	10.96 ± 0.31^a

4. Discussion

In aquaculture, a primary goal of genetic selection is growth improvement of economically important species. Growth traits are physiological functions under the control of several genes and regarded as a phenotype under the control of quantitative trait loci (Jung et al., 2013). Genotyping animals for all the genes encoding a polygenic trait seems impractical, thus, it is more realistic to focus on only a few genes having effects that account for a significant part of the genetic variation in growth traits (Dekkers, 2004; Zhang et al., 2009).

SNP markers in candidate genes can be treated as similar to other bi-allelic co-dominantly segregated DNA markers. The advantage of coding SNPs is that they are located in regions that code for functionally important proteins. Therefore, they are more likely to be near QTLs that affect commercially important traits (Glenn et al., 2005; Lyons and Li, 2002; Tao and Boulding, 2003).

In *P. monodon*, single nucleotide polymorphism by expressed sequence tags (SNP by EST, SBE) was developed by amplification of 102 previously identified ESTs in its ovaries and hemocytes. A total of 48 successfully amplified genes were further characterized by SSCP analysis. Of which, and 44 gene segments were polymorphic. The full-length of *ribophorin I* was successfully characterized by RACE-PCR. Semi-quantitative RT-PCR of *ribophorin I* were carried out and significantly differences on the expression leves of *ribophorin I* ($P < 0.05$) during ovarian and testicular development of *P. monodon* broodstock. Although the preliminary study did not reveal significant relationship between SNP (SSCP patterns) and levels of *ribophorin I* expression in the limited sample size of *P. monodon* broodstock, this approach demonstrates the possibility to further test for relationships between SNP of candidate genes allied with reproduction and growth (degrees of gene expression by the wild type/mutant alleles and reproductive or growth performances) of *P. monodon* (S. Klinbunga, unpublished data).

Several factors influence the growth rate in aquaculture species. The important step in genetic improvement of economically important species is isolation and characterization of particular genes (loci) functionally associated with phenotypic variations in growth (Jung et al., 2013). In the present study, SSCP analysis was applied to determine SNP in the amplified *PmXbp1* gene segment, and the results indicated that polymorphism of *PmXbp1* was associated with growth-related parameters of *P. monodon*. Nucleotide sequences in different individuals of *P. monodon* juveniles were examined.

Although analysis of SNP by DNA sequencing is straightforward, simplification of the detection method of informative phenotypic-related SNPs is another important issue. A relatively simple and cost-effective method for genotyping of SNPs is of interest. It requires specific discrimination of wild type and mutant alleles for the point mutation detection. Allele-specific PCR can be used for this purpose. In the present study, real-time PCR-based PASA was successfully developed to facilitate the detection of $G>A_{76}$ SNP located in the fragment amplified by PmXbp1-F/R primers.

A single SNP (positions 76 of the amplified fragment) was found in four families of the domesticated G7 samples. Growth parameters of shrimp possessing different SNP genotypes were statistically analyzed and revealed potential association with growth traits of *P. monodon* in this study. Juveniles with G/G_{76} showed a better growth performance than those carrying G/A_{76} genotype. Accordingly, a SNP at this position could be used for initial elimination of broodstock that may provide slow-growth juveniles from those that may provide better growth juveniles.

Genetic improvement and other biotechnological applications are crucial to the future for a sustainable development of *P. monodon* cultivation. Our findings about the relationship between growth parameters and SNP genotypes of *PmXbp1* can further be applied to assist the ongoing domestication and genetic improvement program of *P. monodon*. Although our results indicate positive association, it must be kept in mind that shrimp growth is a discontinuous process complicated by molting cycles (Zhuo et al., 2017). Thus, additional research is necessary to eliminate confounding factors including stage and age-dependence, sex differences and environmental conditions before definitive conclusions can be unambiguously made.

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Differential Expression of *X-box Binding Protein 1* following Ammonia Stress in the Pacific White Shrimp *Litopenaeus vannamei*

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Abstract

Intensive culture systems are currently used for farming of the Pacific white shrimp *Litopenaeus vannamei*. Ammonia is the main product from shrimp wastes and uneaten food which causes oxidative stress in cultured shrimp through increasing reactive oxygen species (ROS). Here, effects of ammonia on the expression of *X-box binding protein 1* (*LvXbp1*), one of the key gene functionally related with endoplasmic reticulum stress (ER) stress, in two-month-old *L. vannamei* were examined. Juvenile shrimp (the body weight of 8-10 g) were treated with different concentrations of ammonia (control and 10 mg/l ammonia-N) for 72 h, Hemocytes and hepatopancreas of treated juveniles were collected at 0, 6, 12, 24, 48 and 72 hours post treatment (hpt; $N = 6$ for each group). The expression level of *LvXbp1* in hemocytes of the control shrimp was not significantly different from that of shrimp treated with 10 mg/l ammonia-N at 0, 12, 24, 48 and 72 hpt ($P > 0.05$). However, its expression in the former was significantly greater than that of the latter at 6 hpt ($P < 0.05$). In contrast, the expression level of *LvXbp1* mRNA in hepatopancreas of juveniles exposed to 10 mg/l ammonia-N was significantly greater than that of the control at 12-48 hpt. Results clearly revealed effects of ammonia stress on expression of *LvXbp1* mRNA in hepatopancreas of *L. vannamei*.

Keywords: Pacific white shrimp; *Litopenaeus vannamei*; Ammonia *Xbp1*; Gene expression; Quantitative real-time PCR

1. Introduction

The Pacific white shrimp (*Litopenaeus vannamei*), a native penaeid species of the Pacific coast of Mexico, has become one of the most important shrimp species in aquaculture worldwide (Meehan et al., 2003). Domesticated *L. vannamei* has been introduced to Thailand

as a new cultured species and initially contributed approximately 20,000 MT (7.4%) of the cultured production in 2002. It contributes approximately 98% of total shrimp production with the exported shrimp in Thailand at present (Limsuwan, 2004; Munkongwongsiri et al., 2017; Wyban, 2007).

Intensive culture systems are used to increase the farmed production of *L. vannamei*. However, it results in accumulation of toxic substances from waste products of the shrimp and uneaten food. Combining with the variable and deteriorated aquaculture environments, the toxicity from the degraded culture factors may play an important role in the high mortality of shrimp, which has significantly affected the yield and quality of *L. vannamei* (Chen et al., 2015; Lu et al., 2016).

Ammonia is the main product of protein catabolism in the aquatic system. It is generated mainly in the mineralization process of organic wastes such as unconsumed feed and feces (Chen et al., 2012; Ren et al., 2015) and is a toxic substance in aquaculture water. The increased concentrations of ammonia could be accumulated in the body fluids of aquatic animals, which lead to growth reduction, tissue erosion and degeneration, immune suppression and high mortality (Li et al., 2014). Ammonia has also been reported to cause oxidative stress in organisms through increasing the concentration of reactive oxygen species (ROS) (Hegazi et al., 2010; Murthy et al., 2001).

In order to maintain the homeostasis, organisms employ an antioxidant defense system to eliminate ROS to counteract oxidative stress and prevent oxidant damage. It has been reported that the antioxidant enzyme activity could be induced by low concentrations of stressors. However, the antioxidant response was not sufficient to prevent oxidative damage from the increasing concentrations and exposure time of pollutants. As a result, the excessive level of stress-induced ROS could damage the biomacromolecules including DNA fragmentation and finally lead to the cell dysfunctions (Livingstone, 2001).

Progress in genetic and biotechnology researches in penaeid shrimp has been slow because a lack of knowledge on mechanisms controlling their production traits (Benzie, 1998; Coman et al., 2007; Withyachumnarnkul et al., 1998). Selection for increased juvenile growth rates and stress tolerance is feasible in shrimp. Breeding stocks with high stress-resistance and/or growth rate is of particular interest to shrimp breeders (García et al., 2009). Many stress-related genes have been reported to be upregulated upon induced with toxic substances (Podrabsky and Somero 2004; Schwimmer et al., 2006; Voolstra et al., 2009). However, details of stress-related genes in shrimp are limited. Understanding mechanisms and functions of these genes would provide a new tool for determining health status of shrimp and for understanding the biological and molecular processes to improve stress tolerance in *L. vannamei*.

In the present study, expression profiles of a stress-related genes, *X-box binding protein 1,* in hemocytes and hepatopancreas of *L. vannamei* was analyzed after the shrimp were exposed with different ammonia levels at the salinity of 15 ppt. The expression profiles of *LvXbp1* in these tissues were illustrated.

2. Materials and Methods

2.1 Experimental animals and ammonia stress treatment

A full-sib family was generated from the founder population (called PS3). Juvenile shrimp (first generation called CES3; N = 350) were obtained. Shrimp were acclimated at the laboratory conditions (28 ± 1 ºC, 15 ppt salinity, natural light) for 7 days. Ammonia stress test on shrimp was conducted using a static water system. The control (no supplementation) and 10

mg/l ammonia-N were prepared in 200-l rectangular tanks. Shrimp (N = 108) were separated into two groups and each group was transferred into each ammonia treatment tank. The experiment was carried out for 72 hours and specimens from each tank are collected at 0, 6, 12, 24, 48 and 72 hours post treatment (hpt, N = 9 for each time interval). During the experimental period, shrimp were feeding a commercial diet twice a day without water exchanges. Water parameters (total ammonia, TAN); nitrite (Strickland and Parsons, 1972) and nitrate (Greenberg et al., 1992) were measured. The amount of ammonia were adjusted daily to 10 mg/l ammonia-N for the treatment group.

For examination of effects of ammonia stress on gene expression analysis, hemolymph was collected from shrimp using 10% sodium citrate as an anticoagulant and centrifuged at 1000 g for 5 min. The supernatant was discarded and hemocytes were used for subsequent total RNA extraction. Hepatopancreas of each individual was dissected and immediately placed in liquid nitrogen, and stored at -80°C until used.

2.2 Sequence alignments and primer designed

Nucleotide sequences of *Xbp1* of *L. vannamei* (GenBank JQ265944.1) and other penaeid shrimp were retrieved from GenBank (http://ncbi.nlm.nih.gov). Amino acid and nucleotide sequences of these genes were aligned using ClustalX. Primers (LvXbp1-F: 5′-TTGATGAACTACGGGACCTG-3′ and LvXbp1-R: 5′-GGCTTCGTTGATGTTGCTC-3′) for amplification of a 137 bp cDNA segment were designed using a Primer Premier 5.0 software (Lalitha, 2000).

2.3 Genomic DNA and total RNA extraction and first-strand cDNA synthesis

Genomic DNA was extracted from a piece of the frozen pleopod using a GF-1 Tissue DNA Extraction Kit (Vivantis). Total RNA was isolated from hemocytes and hepatopancreas of *L. vannamei* using TRI Reagent (Molecular Research Center). The quantity of extracted genomic DNA and total RNA was spectrophotometrically estimated. One and a half microgram of DNase I-treated total RNA was reverse-transcribed to the first strand cDNA using an Improm-II™ Reverse Transcription System (Promega). The quality of synthesized template was evaluated by amplification of *EF1-α* from each cDNA template using primers EF1-α-F: ATGGTTGTCAACTTTGCCCC and EF1-α-R: TTGACCTCCTTGATCACACC.

2.4 Determination of gene expression profiles by quantitative real-time PCR

The amplified *LvXbp1* and the reference genes, *β-actin* (primers F: 5′-GAAGTAGCCGCCCTGGTTG-3′ and R: 5′-CGGTTAGCCTTGGGGTTGAG -3′) were cloned into a pGEM-T Easy vector and transformed into *Escherichia coli* JM109 following standard protocols (Sambrook and Russell, 2001). Plasmid DNA of each gene was extracted using a Qiaprep Miniprep Kit (Qiagen) and sequenced for both directions. Standard curves representing 10^3-10^8 copies of *LvXbp1* and *β-actin* were constructed. The target genes and *β-actin* in hemocytes and hepatopancreas of each shrimp were amplified in a 10 µl reaction volume containing 5 µl of 2x LightCycler 480 SYBR Green I Master (Roche), appropriate amount of the first strand cDNA template, 0.3 µM of each gene-specific primer. The thermal profiles were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s. Quantitative real-time PCR of each specimen was carried out in duplicate.

2.5 Data analysis

The relative expression level (copy number of the *LvXbp1*/that of *β-actin*) between shrimp from different exposure time were statistically tested using an independent t-test. Differences were considered to be significant if $P < 0.05$.

3. Results

3.1 Establishment of a full-sib family of L. vannamei

A full-sib families (Family 146) was established by natural mating of a PS3 female with a PS3 male. This family is the first generation of genetically improved *L. vannamei*. Nauplii were reared at the Thai Union Hatchery Co. Limited, Phangnga until they reached the postlarval (PL) stage and PL12 were transferred to the new tank and further cultivated until the PL67 stage. Three hundred fifty individuals were sampled. One hundred and eight juveniles (average body weight = 7.72 ± 1.15 g and total length = 9.71 ± 0.59 cm) were subjected to ammonia stress treatment described previously.

3.2 Ammonia stress treatment and preparation of the first-strand cDNA template

Specimens were collected at 0, 6, 12, 24, 48 and 72 hours post treatment (hpt) with 10 mg/l ammonia-N. Shrimp in the control treatment (without ammonia addition and water exchanges) were also collected at the same time intervals. No shrimp died during the treatment period.

Total RNA was extracted from hemocytes and hepatopancreas of each shrimp and the quality are acceptable for further analysis (Fig. 1). After DNase I treatment, the digested total RNA was reversed-transcribed. The quality of synthesized template was evaluated and *EF1-α* was successfully amplified from all examined template (Fig. 2). The first strand cDNA template was kept at -20°C until required.

3.3 Expression of LvXbp1 upon ammonia treatment

The expression level of hemocytes *LvXbp1* of the control group and that of shrimp treated with 10 mg/l ammonia-N was comparable at 0, 12, 24, 48 and 72 hpt ($P > 0.05$). However, the *LvXbp1* mRNA in the former was significantly greater than that of the latter at 6 hpt ($P < 0.05$; Fig. 3A).

In hepatopancreas, the expression of *LvXbp1* mRNA in juveniles exposed to unchanged culturing water was not significantly different from that of juveniles exposed to 10 mg/l ammonia-N for 0 and 6 hpt. A greater expression level of *LvXbp1* was observed in the latter group at 12-48 hpt. However, its expression level in the control shrimp was greater than that of the treatment at 72 hpt ($P < 0.05$; Fig. 3B).

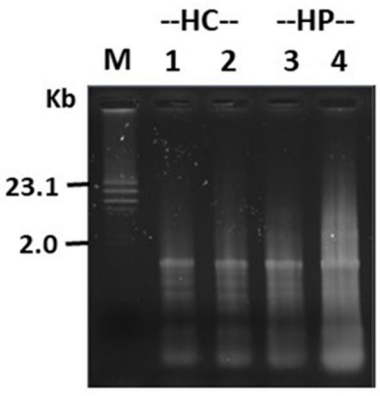

Fig. 1 Examples of total RNA extracted from hemocytes and hepatopancreas of juvenile *L. vannamei.* Lane M = Lambda DNA/*Hin*d III marker, Lanes 1-2 = total RNA of hemocytes, Lanes 3-4 = total RNA of hepatopancreas. HC = hemocytes and HP = hepatopancreas.

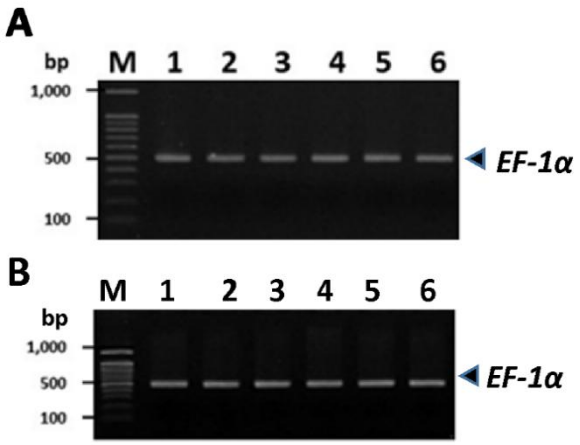

Fig. 2 Amplification of *EF1-α* to examine the quality of synthesized first-strand cDNA of hemocytes (lanes 1-6, A) and hepatopancreas (lanes 1-6, B). Lane M = 100 bp DNA marker.

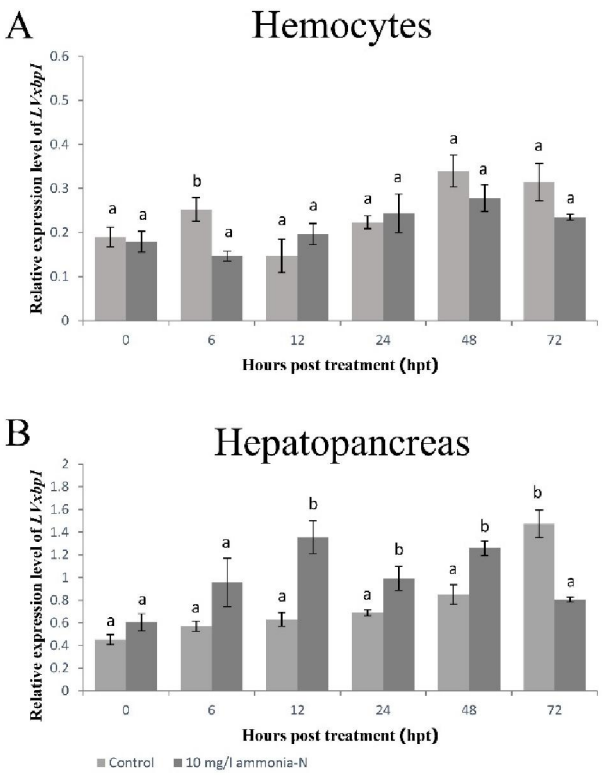

Fig. 3 Histograms illustrating relative expression levels of *LvXbp1* in hemocytes (A) and hepatopancreas (B) of *L. vannamei* exposed to a normal laboratory culture condition and 10 mg/l ammonia-N. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$). Error bars indicate the standard error of the mean (SEM).

4. Discussion

Ammonia toxicants are separated into two types: total ammonia-N and NH_3-N. The acute toxicity tests of ammonia-N and NH_3-N have been widely studied in different developmental stages of penaeid shrimp including larvae (Chin and Chen 1987), juveniles (Chen and Lei 1990; Chen and Lin, 1991) and adolescents (Chen et al., 1990). In penaeid shrimp, the values of 96 h LC50 of NH_3-N ranged between 0.87 mg/l in *M. ensis* (Nan and Chen, 1991) and 2.47 mg/l in *P. chinensis* (Chen and Lin, 1992). Acute toxicity tests in most of the previous studies were conducted using a static renewal condition with water exchange. Salinity is one of the major factors that can influence stress tolerance in shrimp. Chen and Lin (1992) reported that toxicity of ammonia increased as salinity decreased. Inconsistency of LC_{50} values can be seen between studies, indicating a significant effect of various experimental conditions and different shrimp status. Therefore, it is necessary to perform acute toxicity test under similar experimental conditions to be able to make proper comparisons.

Lu et al. (2016) performed comparative transcriptome analysis between ammonia-challenged and control groups from the same family of *L. vannamei* to identify the key genes and pathways response to ammonia stress. In total, 136 significantly differentially expressed genes (DEG) that have high homologies with the known proteins in aquatic species were identified. Among which, 94 genes (e.g., X-box binding protein 1, lactate dehydrogenase, chitinase 1 precursor, alpha-glucosidase, ecdysteroid receptor E75, cyclin A, cyclin B, cytochrome P450, cytochrome c, glucose transporter 1 and phosphoenolpyruvate carboxykinase etc.**)** were reported potentially related to immune function, and the rest of the genes were involved in apoptosis, growth, molting, and osmoregulation. Fourteen GO terms and 6 KEGG pathways were identified to be significantly changed by ammonia stress. In these GO terms, 13 genes have been studied in aquatic species and 11 of them (e.g. thrombospondin protein, ovarian peritrophin, G protein-coupled receptor, penaeidin-2a, cation-independent mannose-6-phosphate receptor and integrin) were reported potentially involved in immune defense and two genes (chitinase and ecdysteroid receptor E75) were related to molting. In the significantly changed KEGG pathways, 7 DEGs (cytochrome P450, mas, alpha 2 macroglobulin, CYP2, cytosolic phospholipase A2, serine/threonine-protein phosphatase and prophenol oxidase activating enzyme) were identified. In addition, expression of 6 genes in nitrogen metabolism pathway (glutamate synthase, glutamate decarboxylase, glutaminase kidney isoform, aminophosphoribosyl transferase, D-aspartate oxidase and omega-amidase NIT2) and two genes in alanine, aspartate and glutamate pathway (carbonic anhydrase 1 and 3) were affected by ammonia stress. The results provided molecular level support for the previous finding on the detrimental effects of ammonia stress in shrimp.

In this study, the expression profiles of *LvXbp1* in response to ammonia stress treatment were examined in two-month-old *L. vannamei*. Hemocytes and hepatopancreas of juveniles were collected at different time intervals after treatment. The expression of hemocytes *LvXbp1* between the control and the treatment groups was not significantly different during the experimental period ($P > 0.05$) except at 6 hpt. In hepatopancreas, the expression of *LvXbp1* in juveniles exposed to unchanged culturing water for 0 and 6 h was not significantly different from that of juveniles treated with 10 mg/l ammonia-N. Longer exposure to ammonia resulted in an increase expression level of *LvXbp1* ($P < 0.05$). Results clearly revealed the positive responses of *LvXbp1* in hepatopancreas following ammonia stress treatment.

Recently, the full-length cDNA of *PmXbp1* in the giant tiger shrimp, *Penaeus monodon* was identified. It was 1762 bp in length containing an ORF of 855 bp corresponding to 284 amino acids. Single nucleotide polymorphisms (SNPs) in *PmXbp1* (185 bp) were examined in

domesticated 3-month-old juveniles (average body weight and total length = 12.32 ± 5.13 g and 11.30 ± 1.58 cm, respectively, N = 162) by single-strand conformational polymorphism (SSCP) analysis. Association between SSCP patterns of *PmXbp1* and growth-related parameters was found where the average body weight (15.07 ± 5.04, N = 44) of juveniles carrying pattern A (corresponding to a T/T_{27} SNP) was significantly greater than that (11.37 ± 4.80, N = 116) of juveniles carrying pattern B (corresponding to a T/C_{27} SNP). The relative expression levels of *PmXbp1* in the hepatopancreas of juveniles carrying different SSCP/SNP genotypes were significantly different (A > B; $P < 0.05$). In addition, the level of *PmXbp1* in shrimp exhibiting a greater growth performance (22.08 ± 2.97 g and 13.60 ± 0.56 cm; N = 11) was significantly lower than that with a lower growth performance (5.00 ± 0.89 g and 8.60 ± 0.98 cm; N = 8) ($P < 0.05$). Comparing the information from this and the previously studies, *PmXbp1* seems to play an important role in growth of shrimp. High concentration of ammonia induced the expression of *LvXbp1* which may readily affect growth of cultured *L. vannamei*. Accordingly, chronic effects of ammonia stress on growth of cultured *L. vannamei* should be further studies.

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Effect of Extenders and Cryoprotectants on Viability of Spermatogonia- and Oogonia-like Cells of Striped Catfish (*Pangasianodon hypophthalmus)*

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Abstract

Cryopreservation of germ cells in fish provides a useful tool for preserving genetic resources for further restoration via germ cell transplantation. The ultimate goal of our study is to develop a cryopreservation method for spermatogonia and oogonia of striped catfish (*Pangasianodon hypophthalmus*). Here, effects of three extenders; Calcium free-Hanks' balanced salt solution, extender of rainbow trout, and Leibovitz's L-15 medium (L-15) and two cryoprotectants; dimethyl sulfoxide (DMSO) and ethylene glycol, on the viability rate were examined. The immature testis or ovary was collected from juvenile fish and frozen by gradually decreasing the temperature at a rate of 1°C/min until -80°C. Subsequently, the frozen testis or ovary was transferred to liquid nitrogen. The cryopreserved testis was dissociated, and the viability of spermatogonia- and oogonia-like cells were determined using trypan blue staining. The highest viability rate of cryopreservation of spermatogonia- and oogonia-like cells was achieved by using L-15 supplemented with DMSO.

Keywords: Cryopreservation; Spermatogonia; Oogonia; Striped catfish; *Pangasianodon hypophthalmus*

1. Introduction

Several fish species have been in danger of extinction, which threatens the global biodiversity of fish. The cryopreservation of the fish gamete has provided a useful tool for long-term preservation of fish genetic resources (Kainin et al., 2014; Mengumphan et al., 2010; Rani et al., 2014). However, the process of preserving fish eggs and embryos has not yet been employed practically. Recently, the cryopreservation of gonadal tissues, including the testis and ovary, was demonstrated in several fish species as an alternative technology for long-term preservation of fish genetic resources (Lee et al., 2013 and 2016; Marinovic et al., 2017; Psenicka et al., 2016). Cryopreservastion of spermatogonial stem cells was demonstrated (Lee et al., 2013).

Furthermore, transplantation of germ cells including spermatogonia and oogonia has recently been developed in several fish species and demonstrated to be an effective technology

for the establishment of surrogate broodstock (Farlora et al., 2014; Morita et al., 2012; Okutsu et al., 2006; Takeuchi et al., 2009; Yazawa et al., 2010). In addition, the surrogate broodstock was shown to produce donor-derived offspring, suggesting that this technology would be a powerful tool for seed production that can facilitate aquaculture as well as conservation (Yoshizaki 2018). Therefore, the combination of cryopreservation of gonadal tissue containing spermatogonia or oogonia and germ cell transplantation techniques would offer effective tools for the preservation and restoration of the genetic resources of fish (Lee et al., 2013 and 2016).

The family Pangasiidae comprises several economically important fish species, particularly the striped catfish (*Pangasianodon hypophthalmus*). Some members of this family have been classified as endangered species, such as the Mekong giant catfish (*P. gigas*) and the Chao Phraya giant catfish (*Pangasius sanitwongsei*). To develop a preservation approach for fish genetic resources, the present study aimed to determine the preliminary information about cryopreservation of the whole testis and ovary, which contain high proportion of spermatogonia and oogonia of *P. hypophthalmus*, respectively.

2. Materials and Methods

2.1 Experimental fish

Striped catfish (*P. hypophthalmus*) were maintained at the Suranaree University of Technology Farm (SUT Farm); Nakhon Ratchasima, Thailand. The experimental fish (50-80 g; $N = 150$/treatment) were maintained in cages (2 x 2 m^2) located in an earthen pond (400 m^2). Immature ovaries (ovarian weight = 0.0438 ± 0.0193 g, gonadosomatic index, GSI = 0.0799 ± 0.0494%) and testes (testicular weight = 0.0101 ± 0.0054 g, GSI = 0.0175 ± 0.0074%) were collected and incubated in Leibovitz's L-15 medium (Gibco) before cryopreservation.

2.2 Cryopreservation

The composition of three extenders including Calcium free-Hanks' balanced salt solution (CF-HBSS), extender of rainbow trout (extender RT) (Lee et al., 2013), and L-15 medium is shown in Table 1. Two cryoprotectants; dimethyl sulphoxide (DMSO) and ethylene glycol at a concentration 1.3 mM were mixed with extenders to prepare the cryomedium and incubated at 4°C. For each cryoprotectant tested, either testis or ovary was transferred into a 1.8 ml cryovial (SPL LIVE SCIENCES) containing 0.5 ml cryomedium. Cryovials were equilibrated on ice for 60 min and gradually cooled at a rate of -1°C/min for 90 min using a Bicell plastic freezing container (Nihon Freezer, Japan) incubated in a -80°C refrigerator. After freezing, the samples were plunged into liquid nitrogen and stored for at least 1 week. When required, samples were thawed at 10°C for 8 min and rehydrated on ice using the L-15 medium (5 min/time).

2.3 Determination of viability rate

To obtain single cells, fresh or frozen gonads (testis and ovary) were dissociated to individual cells using 0.4% Collagenase H and 0.03% Dispase II, as described by Morita et al. (2012). Viability rates were determined using the trypan blue staining assay. The cell suspensions were incubated in 0.2% trypan blue solution for 5 min. Viability (%) was calculated as the percentage of trypan blue negative cells found among the total observed cells.

2.4 Statistical analysis

Statistical analysis was performed using one-way ANOVA statistical test (R-software). When significant differences were found among the groups, Tukey range test was conducted to rank the groups. In addition, t-test analysis was performed to evaluate the difference between the values of fresh and frozen tissues. Differences were considered significant when $P < 0.05$.

Table 1 Composition of extenders used in the present study

Composition	Extender		
	CF-HBSS[1] (mM)	Extender RT[2] (mM)	L-15
NaCl	152.12	375.48	-
KCl	5.9	7.28	-
$MgSO_4 \cdot 7H_2O$	0.89	-	-
$Na_2HPO_4 \cdot 7H_2O$	0.48	3.82	-
KH_2PO_4	0.51	23.10	-
$NaHCO_3$	4.64	-	-
Glucose	6.16	-	-
HEPES	-	55.27	-
Sodium Pyruvate	-	3.64	-
$CaCl_2 \cdot 2H_2O$	-	2.6	-
$MgCl_2 \cdot 6H_2O$	-	1.4	-
L-15	-	-	13.7 g
DI Water	-	-	1 L
pH	7.6	7.8	7.8

[1] Mongkonpunya et al. (1995)
[2] Lee et al. (2013)

3. Results

Juvenile male and female striped catfish were used in the present study. The testis of the juvenile fish contained spermatogonia or type A spermatogonia (Fig. 1A), and its ovary comprised oogonia and oocytes (Fig. 1B). To obtain spermatogonia and oogonia, a fresh testis and ovary were dissociated, respectively. The viability rates of spermatogonia and oogonia were 91.73% and 92.80%, respectively (Fig. 2 and Tables 2 and 3).

Cryopreservation of spermatogonia and oogonia were performed using the combination of three extenders (CF-HBSS, extender RT, and L-15) and two cryoprotectants (DMSO and EG). Comparing with the fresh testis and ovary, the frozen spermatogonia and oogonia had significantly lower viability rates ($P < 0.05$) (Table 2). For cryopreservation of spermatogonia, both extenders and cryoprotectants had effects on the viability rate of spermatogonia. However, no interaction effects were observed. The significantly highest viability rate of frozen spermatogonia was observed with the extender L-15. Moreover, DMSO showed the highest viability rate for frozen spermatogonia. Effects of extenders, cryoprotectants, and their interactions were also observed in cryopreservation of oogonia. Among combinations of extender and cryoprotectant, the extender RT with cryoprotectant EG showed the lowest viability rate of oogonia. The other combinations of extender and cryoprotectant were found to have similar effects.

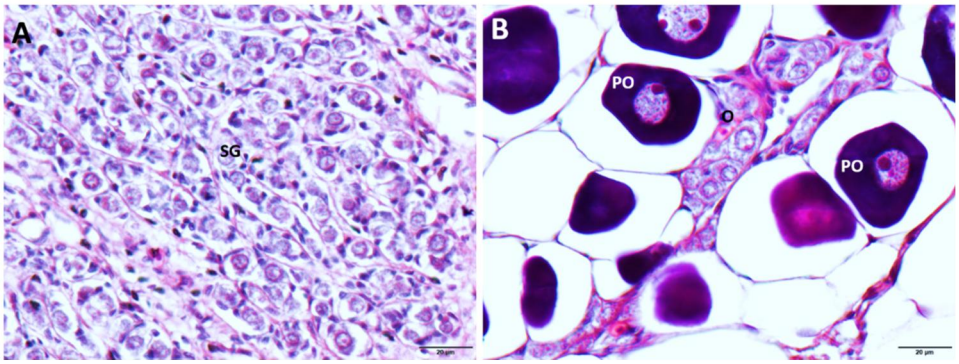

Fig. 1 Histological characterization of the testis (A) and ovary (B) of juvenile fish. Serial transverse sections of the immature testis and ovary were performed and stained with haemotoxylin and eosin. PO, previtellogenic oocyte; O, oogonia; SG, spermatogonia. Scale bars represent 20 µm.

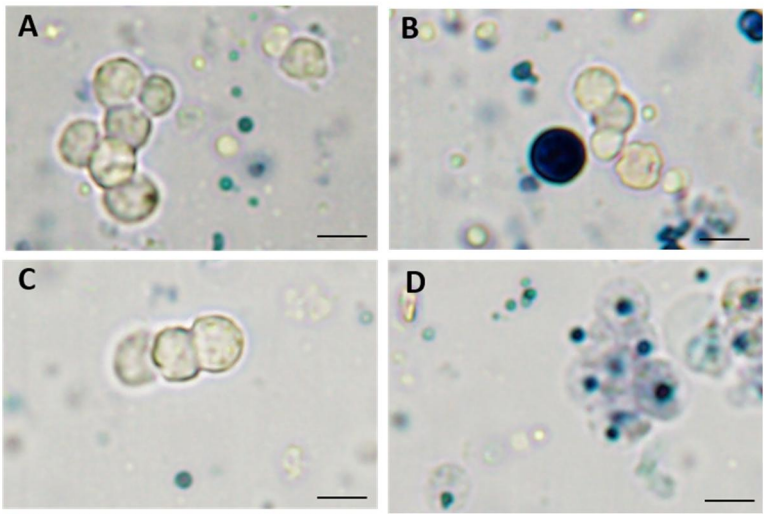

Fig. 2 Trypan blue exclusion assay of frozen spermatogonia- and oogonia-like cells. Spermatogonia- (A-B) and oogonia-like (C-D) cells stained with blue color were considered nonviable, whereas viable cells were not stained. Scale bars = 10 µm.

Table 2 Viability of cryopreservation of spermatogonia-like cells (mean ± SD, $N = 5$)

Extender	Cryoprotectant	Viability
Fresh		91.73 ± 11.45^{A}
CF-HBSS	DMSO	60.67 ± 4.94^{abB}
	EG	43.99 ± 5.48^{cB}
RT extender	DMSO	61.33 ± 1.83^{abB}
	EG	50 ± 3.33^{bcB}
L-15	DMSO	72.67 ± 9.83^{aB}
	EG	58 ± 10.7^{bB}

Means with different lower-case superscripts differ significantly from each other ($P < 0.05$). Different capital superscripts denote significant differences between fresh and frozen tissues ($P < 0.05$).

Table 3 Viability of cryopreservation of oogonia-like cells (mean ± SD, $N = 5$)

Extender	Cryoprotectant	Viability
Fresh		92.8 ± 8.07^{A}
CF-HBSS	DMSO	51.33 ± 10.7^{aB}
	EG	46 ± 13.82^{aB}
Extender RT	DMSO	58.67 ± 19.24^{aB}
	EG	18 ± 5.58^{bB}
L-15	DMSO	66 ± 7.07^{aB}
	EG	61.33 ± 15.74^{aB}

Means with different lower-case superscripts differ significantly from each other ($P < 0.05$). Different capital superscripts denote significant differences between fresh and frozen tissues ($P < 0.05$).

4. Discussion

Cryopreservation of the whole testis and ovary in fish offers a tool for preservation of genetic resources. Recently, germ cell transplantation in fish has been developed for several aquaculture species (Lee and Yoshizaki, 2016). The combination of cryopreservation of germ cells and germ cell transplantation in fish presents a biotechnological tool to preserve and restore fish, thereby facilitating the cryopreservation of endangered fish species. For instance, spermatogonial and oogonial cell transplantation was developed in salmonids and demonstrated to provide donor-derived offspring. In the present study, the cryopreservation of spermatogonia and oogonia of striped catfish was conducted for possible applications of germ cell transplantation in pangasiid fish.

As the first step of the cryopreservation technique, we determined the appropriate extender and cryoprotectant for freezing the whole testis and ovary. Generally, the extender has a significant effect on the success of cryogenic preservation of cells. The composition of the extender and its osmolality influence the viability of frozen cells. Several extenders have been used for cryogenic preservation of the testis and ovary in fish. For example, phosphate buffered saline (PBS) with 0.5% bovine serum albumin and 50 mM glucose was an appropriate extender for the frozen ovary and testis of Siberian sturgeon, *Acipenser baerii* (Psenicka et al., 2016). Moreover, this extender was suitable for cryopreservation of the testis of tench, *Tinca tinca* and goldfish, *Carassius auratus* (Marinovic et al., 2017). L-15 was revealed to be an appropriate extender for cryopreservation of the testis of the tiger puffer, *Takifugu rubripes* (Yoshikawa et al., 2018). Our results showed that both frozen spermatogonia- and oogonia-like cells had the highest viability rates with the extender L-15. For cryopreservation of the

sperm of pangasiid fish, CF-HBSS was demonstrated to be an appropriate extender in several species, such as *Pangasianodon gigas*, *Pangasius bocourti*, and *Pangasius suchi* (Kainin et al., 2014; Mengumphan et al., 2010; Rani et al., 2014). However, CF-HBSS was not a suitable extender for cryopreservation of spermatogonia- an oogonia-like cells in the present study. An extender RT was demonstrated to be an appropriate extender for cryopreservation of the testis, ovary, and sperm in rainbow trout, *Oncorhynchus mykiss* (Lahnsteiner et al., 1996; Lee et al., 2013 and 2016). Nevertheless, the percentage of viable spermatogonia- and oogonia-like cells from this extender was inferior to that of L-15.

Our study showed that DMSO was an appropriate cryoprotectant for the cryopreservation of both spermatogonia- and oogonia-like cells of *P. hypophthalmus*. From previous studies, DMSO was revealed to be an optimum cryoprotectant in cryopreservation of the testis in several fish species, including *O. mykiss*, *T. tinca*, *C. auratus*, and *T. rubripes* (Lee et al., 2013; Marinovic et al., 2017; Yoshikawa et al., 2018). In addition, DMSO was demonstrated to be a suitable form of cryopreservation for the ovary of *O. mykiss* (Lee et al., 2016).

In conclusion, our preliminarily study revealed that L-15 and DMSO were an appropriate extender and cryoprotectant, respectively, for the cryopreservation of both spermatogonia- and oogonia-like cells in the striped catfish. The basic information obtained from this study will be further applied for the development of a preservation approach for genetic resources of *P. hypophthalmus*.

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Identification and Treatment of *Fusarium* Isolated from Black Spots on the Cuticle of Pacific White Shrimp *Litopenaeus vannamei*

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Abstract

Pacific white shrimp (*Litopenaeus vannamei*) is the most cultivated shrimp species in Thailand at present. Bacterial and viral diseases affect the culture production of this species. In addition, fungal diseases can also affect the cultured shrimp. Here, seven broodstock-sized *L. vannamei* showing black infected lesions on the cuticle at various parts of the body were collected. The portion of the wounds (cuticle and attached tissues) were aseptically dissected and observed under a light microscope. Similar signs of infection with several hyphae-like structures protruding from the cuticle into the tissues underneath and brown pigmentation surrounding the hyphae were observed in all shrimp. Glucose Yeast Peptone (GYP) medium supplemented with ampicillin (250 µg/ml) and chloramphenical (100 µg/ml) was used to isolate the fungi. The 18S ribosomal RNA sequences of representative individuals were determined. The closest matched of most fungal isolates was *Fusarium* which is known fungal pathogen affecting the cuticle and gill of the shrimp. The symptoms are expanding wounds with melanization. Natamycin was applied for the possible treatment of the pathogenic agent. The minimum inhibitory concentration (MIC) of natamycin against cultured *Fusarium* was evaluated and found to be 3.125 µg/ml. In addition, various concentrations of natamycin (0-25 µg/ml) were also tested against bacteria isolated from the pond water. The results indicated no inhibition effect of natamycin on growth of examined bacteria. Our results revealed the effective treatment of natamycin on *Fusarium* with the safe effect on beneficial bacteria in the culture system of *L. vannamei*.

Keywords: Fungi; *Fusarium* sp.; Pacific white shrimp; *Litopenaeus vannamei*; natamycin

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is the most cultivated shrimp species in Thailand at present. However, farming of *L. vannamei* in the region has faced problems from susceptible to severe shrimp pathogens resulting in a decrease of its production. Although bacterial (vibriosis and early mortality syndrome, EMS) and viral diseases (TSV, WSSV and YHV etc.) are the major concern during the juvenile pond growing stages, fungal diseases can also affect the cultured shrimp.

Several fungi are known as shrimp pathogens. In larval stages, three groups including *Lagenidium*, *Siroipidium* and *Haliphthoros* are commonly found. However, *Fusarium* is the most common genus affecting larger shrimp. This fungus may entry into the body through cracks or eroded areas on the cuticle. *Fusarium* may be identified by the presence of canoe-shaped macrocondia that the fungus produced (Johnson, 1989).

The information in the literature confirmed that the genus *Fusarium* was known fungal pathogens for many shrimp species including *L. vannamei, Penaeus monodon, Macrobrachium* sp.*,* and *Homarus* sp. (Fisher et al., 1978). The affected areas included the cuticle and gill of the shrimp, and the symptoms are expanding wounds (cuticular or subcuticular) with melanization. Under the microscope, the wounds are composed of masses of hemocytes; necrotic tissue debris, and hyphae of the fungus (Fisher et al., 1978). *Fusarium* infection is chronic, progressive, and can lead to death of the host. There is no practical method to treat *Fusarium* infection. Fisher et al. (1978) suggested that preventing of infection by eliminating shrimp with heavy infection from the tank, maintaining water quality and practicing good husbandry were the best way to control *Fusarium* infection.

Khoa et al. (2004) isolated *Fusarium incarnatum* from gill lesions of cultured *P. monodon* during 2000-2002 in Nghe An province, Vietnam. Infected shrimp showed typical signs of black gill disease and mortalities about a month prior to harvest. An artificial infection of two isolates was carried out in the kuruma prawn, *Marsupenaeus japonicas* and the pathogenicity of *F. incarnatum* was reported.

In this study, fungi were isolated from broodstock-sized *L. vannamei* showing symptoms of the black shell disease. Molecular phylogenic analysis based on the partial nucleotide sequences of 18S ribosomal DNA (*18S rDNA*) was carried out. Minimum inhibitory concentration (MIC) of isolated fungi against different concentrations of natamycin was examined.

2. Materials and Methods

2.1 External observation of infected shrimp

Seven broodstock-sized white shrimp were collected from the cultured ponds and transported to Aquatic Molecular Genetics and Biotechnology Laboratory, National Center for Genetic Engineering and Biotechnolofy (BIOTEC). All seven shrimp showed black infected lesions on the cuticle at various parts of the body including the carapace, the eyestalk, the tail and/or the last segment of the abdomen. (Fig. 1)

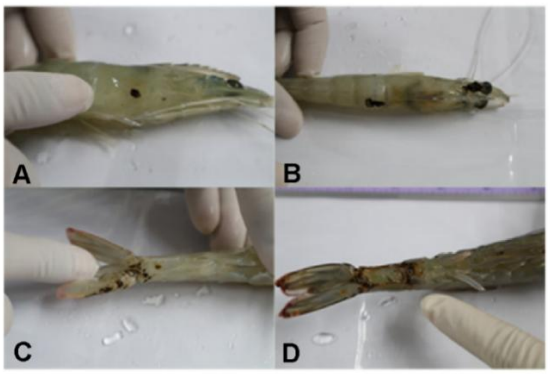

Fig. 1 External observation of *L. vannamei* with symptoms of infection (black spots, A and B and shell erosion, C and D) at various part of the body.

2.2 Microscopic observation of infected wounds and subcuticular muscle

The portion of the wounds on the cuticle was aseptically dissected from infected shrimp and observed as wet mount on a light microscope.

2.3 Isolation of infected fungi and identification of species

A fungal selective medium; Glucose Yeast Peptone (GYP) supplemented with ampicillin (250 µg/ml) and chloramphenical (100 µg/ml) was used to isolate fungi from 4 shrimp individuals (TU1, TU2, TU3, and TU4). After incubation at room temperature for 7 days, circular colonies with filamentous edges were observed. Genomic DNA was extracted from a single colony of fungus using a DNeasy Extraction Kit (Qiagen). The partial *18S rRNA* gene of each fungus colony was amplified by PCR using 18S-EUK581F (5′-GTGCCAGCAGCCGCG-3′) and 18S-EUK1134R (5′-TTTAAGTTTCAGCCTTGCG-3′) primers. The product was cloned into a pGEM-T Easy vector plasmid and transformed into *E. coli* (Sambrook and Russell, 2001). The plasmid containing the partial *18rRNA* gene insert was extracted and sequenced for both directions. Nucleotide sequences were searched for the closest sequence against previously data in GenBank using BlastN (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov).

2.4 Fungal culture, cell preparation and MIC analysis

Pieces of carapace of each infected shrimp were dissected out and placed in 300 µl of a normal saline (0.85% NaCl) solution. The suspension was spread on the GYP agar plate supplemented with ampicillin (250 µg/ml) and chloramphenical (100 µg/ml). After incubation at room temperature for 7 days, 1 ml of the normal saline solution with 0.005% Tween 20 was added and circular colonies with filamentous edges were swopped. The cell suspension was transferred into a culture tube. The optical density at 530 (OD_{530}) was measured and adjusted to 0.15-0.17. The cell suspension was 50-fold diluted with the GYP medium containing ampicillin and chloramphenicol. One hundred microliters of the diluted cell suspension was added to each of 96-well plate. Subsequently, 100 µl of different concentrations of a stock solution of natamycin was added to generate a two-fold serial concentration between 0-25 µg/ml (Pfaller et al., 2002). The culture plate was incubated at 35°C for 48 h. The OD_{530} of treated fungus was measured using a microtiter plate reader.

3. Results and Discussion

All seven shrimp individuals were microscopically examined and showed similar signs of infection. The infected pathogen had a brown string-like shape (hyphae) distributed on the cuticle and into the muscle underneath the cuticle. Upon closer inspection at higher resolution, the string itself was colorless. The brown pigment that was observed surrounded portions of the strings was likely the consequence of melanization around pathogens which is a part of the shrimp immunity reacting to the invading pathogens (Fig. 2).

The size of the strings were approximately 50-100 µm, and can be visualized using 200X to 400X magnification which made this pathogen larger than typical sizes for bacteria. From morphology and the size of examined pathogen, we believed that the disease should be caused by fungal rather than bacterial infection.

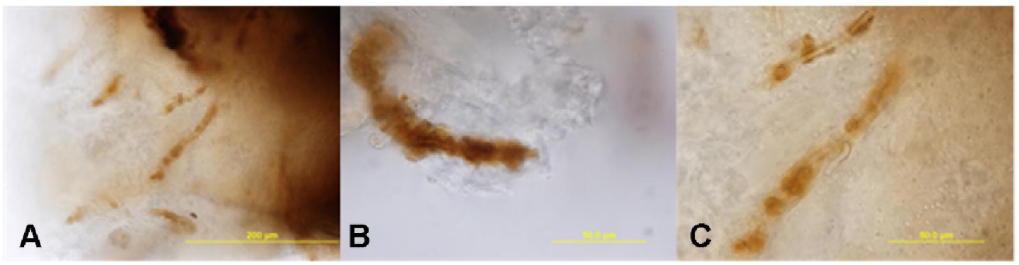

Fig. 2 Microscopic observation (wet-mount) of wounds (A) and subcuticular muscle (B and C) of infected *L. vannamei* broodstock.

We then isolated the fungi from the infection. After incubation at room temperature for 7 days, circular colonies with filamentous edges were observed on the plates. The ability to grow on a fungal selective medium confirms our microscopic observation results on the fungal infection of examined shrimp (Fig. 3)

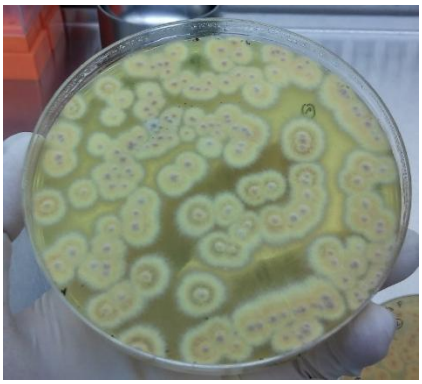

Fig. 3 Colony morphology on the plates of fungi isolated from infected *L. vannamei*.

DNA analysis was carried out to identify the species origin of isolated fungi. The resulting DNA sequences were compared with those in the Ribosomal database. Twelve colonies from four examined shrimp showed closest similarity with either *Fusarium sp.* or *F. oxysporum* while three colonies from two shrimp individuals (TU2 and TU3) exhibited the closest similarity with *Candida parapsilosis*. In addition, one colony isolated from TU3 showed significant similarity with *Boechera divaricarpa* (Fig. 4).

Fusarium is a known fungal pathogen affecting the cuticle and gill of the shrimp. The symptoms are expanding wounds with melanization (Sindermann, 1989). To evaluate the possible use of an antibiotic for treatment of infected shrimp, the MIC of a food-grade natamycin against cultured *Fusarium* was evaluated.

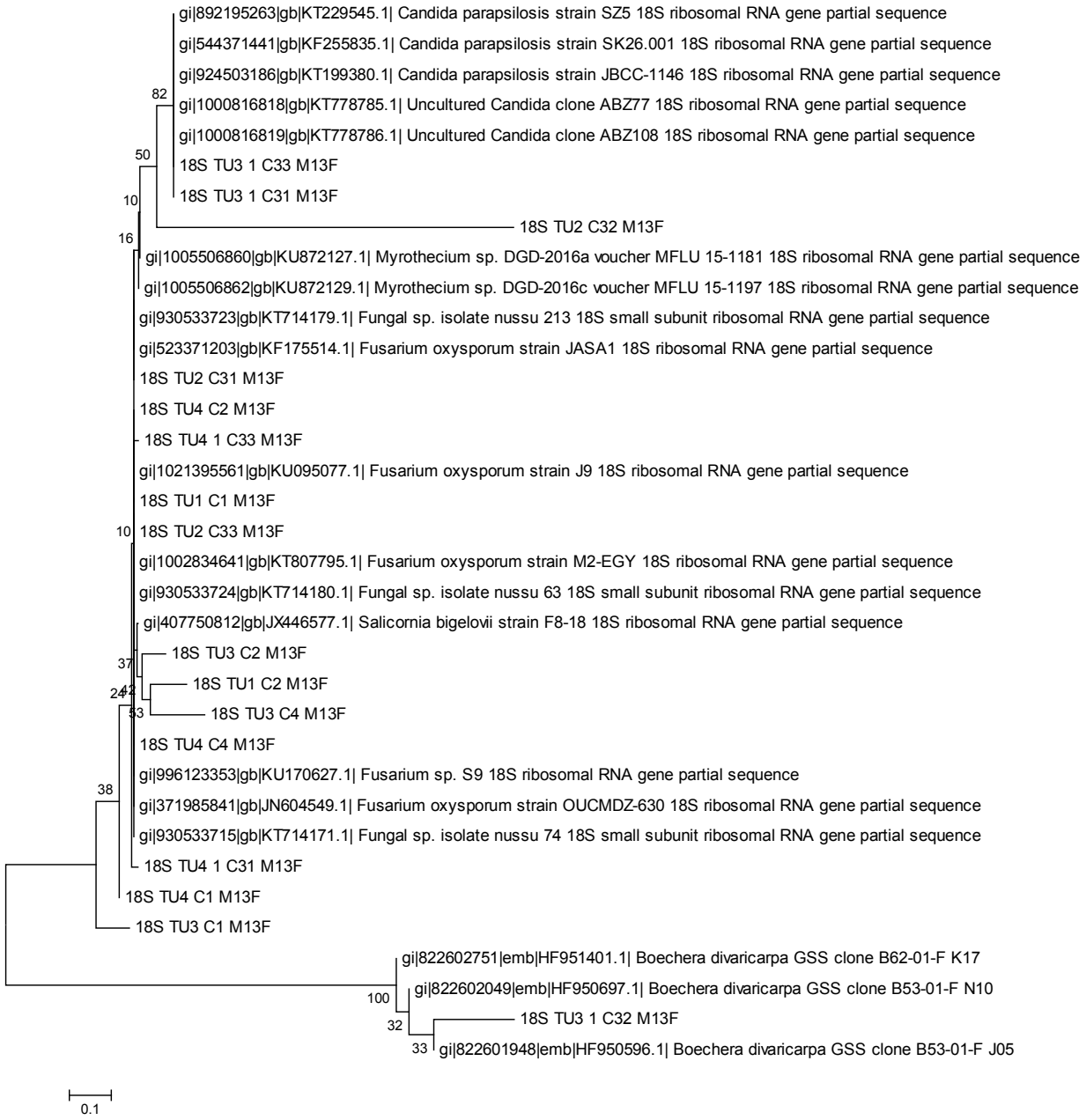

Fig. 4 A phylogenetic tree of *18S rRNA* gene from isolated fungi in this study and reference sequences from the NCBI database. Values at the node represent the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original aligned sequences.

The results clearly indicated that natamycin at the concentration greater than 3.125 µg/ml can effectively inhibit the growth of *Fusarium* isolated from shrimp in the present study (Fig. 5). In addition, the same serial concentrations of natamycin (0-25 µg/ml) were also tested against bacteria isolated from water of the cultured pond. The results indicated no inhibitory effect of natamycin on growth of bacteria at any concentration (data not shown). Therefore, our results revealed the effective treatment of natamycin on *Fusarium* with the safe effect on beneficial bacteria in the culture system of *L. vannamei*.

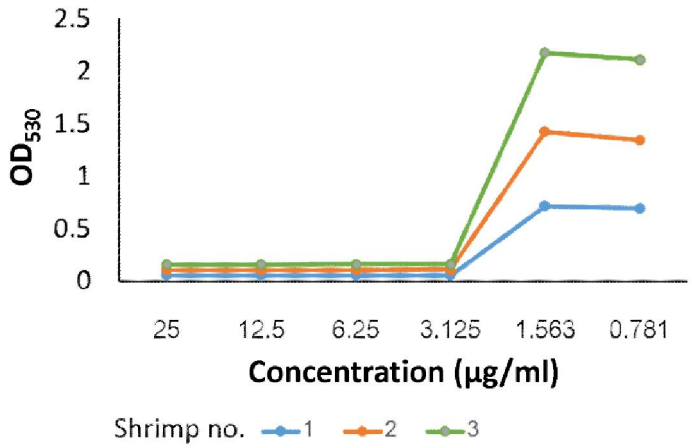

Fig. 5 Effects of different concentrations of natamycin against cultured *Fusarium*.

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