Marine Genomics 22 (2015) 55–61

Contents lists available at ScienceDirect

Marine Genomics

journal homepage: www.elsevier.com/locate/margen

Method paper

Identification of genes involved in reproduction and lipid pathway metabolism in wild and domesticated shrimps

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ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form 3 April 2015
Accepted 5 April 2015
Available online 15 April 2015

Keywords:
Penaeus monodon
Reproductive performance
Lipid metabolism
Gene expression

ABSTRACT

The aims of this study were to identify genes involved in reproduction and lipid pathway metabolism in *Penaeus monodon* and correlate their expression with reproductive performance. Samples of the hepatopancreas and ovaries were obtained from a previous study of the reproductive performance of wild and domesticated *P. monodon* broodstock. Total mRNA from the domesticated broodstock was used to create two next generation sequencing cDNA libraries enabling the identification of 11 orthologs of key genes in reproductive and nutritional metabolic pathways in *P. monodon*. These were identified from the library of de novo assembled contigs, including the description of 6 newly identified genes. Quantitative RT–PCR of these genes in the hepatopancreas prior to spawning showed that the domesticated mature females significantly showed higher expression of the *Pm Elovl4*, *Pm COX* and *Pm SUMO* genes. The ovaries of domesticated females had a significantly decreased expression of the *Pm Elovl4* genes. In the ovaries of newly spawned females, a significant correlation was observed between hepatosomatic index and the expression of *Pm FABP* and also between total lipid content and the expression of *Pm CYP4*. Although not significant, the highest levels of correlation were found between relative fecundity and *Pm CRP* and *Pm CYP4* expression, and between hatching rate and *Pm Nvd* and *Pm RXR* expression. This study reports the discovery of genes involved in lipid synthesis, steroid biosynthesis and reproduction in *P. monodon*. These results indicate that genes encoding enzymes involved in lipid metabolism pathways might be potential biomarkers to assess reproductive performance.

1. Introduction

Over the past decade, many shrimp farmers have switched from farming the Giant Tiger shrimp *Penaeus monodon* to the Pacific white shrimp *Litopenaeus vannamei* which now dominates global shrimp production. One of the key reasons that farmers prefer *L. vannamei* is the comparatively slow progress in the domestication and selective breeding of *P. monodon* (Briggs et al., 2005). Although some advances have been made in domesticating *P. monodon* (Preston et al., 2009, 2010), seedstock production is less efficient than wild broodstock (Arnold et al., 2013; Coman et al., 2013; Marsden et al., 2013) and most *P. monodon* hatcheries continue to rely on wild-caught broodstock (Klinbunga et al., 2010). A broad range of parameters have been used to evaluate reproductive performance in wild and cultured crustacean populations. These include biological (survival, growth–molting, fecundity, spawning and hatching rate), morphometrical (weight and carapace length), histological (mainly ovary and hepatopancreas function–anatomical changes) and biochemical parameters, such as elemental and proximal composition, lipid classes and fatty acids, and digestive enzymes (cf. Arnold et al., 2013; Glencross et al., 2013; Rotllant et al., 2013, 2014). The domestication of *P. monodon* has been constrained, in part, by the lack of basic information about ovarian development and maturation in Penaeid shrimp (Uawisetwathana et al., 2011; Talakhun et al., 2012; Hiransuchalert et al., 2013; Wimuttisuk et al., 2013; Phinyo et al., 2014; Treerattrakool et al., 2014).

In Penaeid shrimp, ovarian development is characterized by the accumulation of a major yolk protein vitellin and the formation of cortical rods in the oocytes. The precursor of vitellin, vitellogenin (Vtg), is synthesized in the ovary and hepatopancreas, transported to the oocytes, and accumulate in the ooplasm as vitellin for utilization as a nutritional source during embryogenesis. After the completion of yolk accumulation, the cortical rods are formed radially around the periphery of the oocyte plasma membrane, and mature oocytes are spawned. Upon spawning, the content of the cortical rods is released around eggs and forms into a jelly-like layer which envelops the eggs. The process is under the control of a neuroendocrine organ in the eyestalk (the X-organ sinus gland complex) (Okumura et al., 2006). Vitellogenin receptor (VgR), vitellogenin inhibiting hormone (VIH) and cortical

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http://dx.doi.org/10.1016/j.margen.2015.04.001
1874-7787/

rod proteins (CRP) have been identified in *P. monodon* (Tiu et al., 2006; Tiu et al., 2008; Treerattrakool et al., 2008, Lehnert et al. unpublished—NCBI) and might be used as indicators for ovarian maturation.

Nutrition plays a substantial role in reproductive processes of Penaeid shrimp, particularly the levels of dietary total and specific lipids. Lipid content has been reported to decrease in the hepatopancreas and transferred to the ovary during maturation (for reviews see Wouters et al., 2001; Glencross, 2009). For *P. monodon* broodstock the dietary total lipid requirement is around 11% of total fed dry matter (Marsden et al., 1997) and the essential cholesterol to 1.7% in juveniles (Smith et al., 2001). Several studies have shown that arachidonic acid (ARA) plays a key role in egg development and spawning in *P. monodon* (Huang et al., 2008; Hoa et al., 2009). The prostaglandins (eicosanoids) are a suite of hormones known to influence a range of physiological processes including reproduction. Arachidonic acid is a precursor in prostaglandin synthesis and is therefore considered to have a further key functional role in reproduction. When prostaglandin biosynthesis was examined in different stages of *P. monodon* ovaries, Wimuttisuk et al. (2013) found that the amounts of prostaglandin F synthase gene transcripts and prostaglandin F2α decreased as the ovaries matured. Therefore, it has been proved that lipid metabolism is a key process in *P. monodon* ovarian maturation.

By contrast to peptide hormones related to reproduction, the study of lipid metabolism requires complex biosynthetic pathways for their synthesis. This in turn relies on the identification of genes encoding these biosynthetic steps, namely fatty acid, eicosanoid and cholesterol biosynthesis. The aim of the study was to discover genes involved in reproduction and lipid pathway metabolism, and to correlate their expression with reproductive performance parameters in wild and domesticated broodstock of *P. monodon*.

2. Material and methods

2.1. Shrimp broodstock origin and rearing conditions, tissue sampling and virus screening

A previous study (Arnold et al., 2013) used cultured wild broodstock (W) caught off the coast of Innisfail (17°53′S, 146°01′E, Queensland, Australia) and eighth generation domesticated (D) *P. monodon* stock originated from wild founder stocks collected from a population off the coast of Weipa (12°48′, 141°32′, Gulf of Carpentaria, Queensland, Australia) to determine reproductive performance in a series of reciprocal cross matings. Taking into account the previous study results for hatching rate, we classified the females into good spawners and poor spawners and we compared them with females just before spawning (I = initial). The characteristics of each group of broodstock are summarized in Table 1. For gene expression analyses we sampled ovaries from the initial group and hepatopancreas for all the groups. To rule out the effects of occasional pathologies in Australian farms, we analyzed hepatopancreas tissues for virus screening; Mourilyan virus and gill associated virus by qPCR following the method of De la Vega et al. (2004) and Rajendran et al. (2006), respectively.

2.2. RNA extraction, gene screening, P. monodon libraries generation and primer design

RNA was extracted from the hepatopancreas and ovary tissues from three to six individuals from each treatment. Total RNA was extracted using RNeasy RNA Extraction Columns and on-column DNase digestion (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. RNA quantity was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA), and subsequently each sample diluted to 100 ng μL^{-1}. The quality of all RNA samples was assessed using the Agilent RNA 6000 Nano Kit and the Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). For the hepatopancreas and ovary next generation sequencing libraries, a pool of RNA extractions from 12 and 10 animals, respectively, to contain 300 μg of total RNA was used. Samples were sent for pyrosequencing using two separate regions of 1/4 plate on a Roche 454 GS − FLX Titanum sequencer (Macrogen, Korea). Results were trimmed removing the low quality data (quality of phred score < 30), 454 adaptors and 50 bp minimum read length, and a partial transcriptome for both ovary and hepatopancreas was de novo assembled using CLC Genomics Workbench software (CLC Bio, Aarhus, Denmark). These libraries were investigated for target genes in shrimp reproduction using the most closely related species in scientific journals and NCBI data bases sequences (Ibarra et al., 2007) and/or lipid nutritional pathways to the Kyoto Encyclopedia of Genes and Genome (KEGG) as fatty acid, steroid and eicosanoid biosynthesis (http://www.genome.jp/kegg/). Putative gene orthologs were identified by BLASTX sequence similarity searches using CLC Main Workbench software (CLC Bio, Aarhus, Denmark). Primers for each specific gene target were designed using PerlPrimer program (Marshall, 2004), using the following conditions: temperature: 58–62 °C, distance between pair of primers: 100–300 pb and, GC%: 45–55%. The list of pairs of primers for each gene is detailed in Table 2.

2.3. Gene expression analysis and quantification

cDNA synthesis was performed on the 26 hepatopancreas and the 10 ovary samples using the SuperScript III First-Strand Synthesis System for RT–PCR (Invitrogen, Carlsbad, CA, USA, Cat. No: 18080-051). An equivalent of 1 μg of total RNA was reverse transcribed following previously published protocols (Callaghan et al., 2010), which incorporated 400 pg of non-endogenous Luciferase RNA (Promega L4561, Venlo, The Netherlands) as an internal control.

Real-time PCR reactions were carried out using 1× SYBR Green PCR Master Mix (Life Technologies Corporation, Carlsbad, CA, USA), 0.2 μmol^{-1} of each primer and the equivalent of 7.5 ng of reverse − transcribed RNA. Amplification cycle conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 40 s at 60 °C, and completed by a dissociation melt-curve analysis. Reactions were set up in triplicate and run on an ABI ViiA7 Real-time PCR system

Table 1
Selected wild (W) and domesticated (D) female broodstock from Arnold et al. (2013) and classification according their hatching rate (I = initial, before spawning; GS = good spawner; BS = bad spawner).

	WI	DI	WGS	WBS	DGS	DBS
N	5	5	3	3	4	6
Ovarian stage[1]	IV	IV	0	0	0	0
Female weight (mg)	140.98 ± 22.19	161.80 ± 26.02	141.57 ± 23.17	151.63 ± 6.80	164.80 ± 33.13	159.40 ± 10.00
Hepatosomatic index	2.96 ± 0.32	2.86 ± 0.25	2.71 ± 0.43	2.74 ± 0.34	2.58 ± 0.21	2.60 ± 0.60
Hatching rate[2] (%)			78.07 ± 11.26	41.10 ± 11.32	62.35 ± 9.29	25.57 ± 5.87
Relative fecundity			4.00 ± 1.09	5.02 ± 1.60	1.77 ± 0.52	2.30 ± 0.86

[1] Ovarian stage following Tan-Fermin & Pudadera (1989).[2]Hatching rate calculation only included the fertilized eggs.