Polychaete consumption increased prostaglandin biosynthesis in female *Penaeus monodon*

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Abstract

The polychaete *Perinereis nuntia* is preferred over commercial feed pellets for boosting ovarian maturation of the female black tiger shrimp *Penaeus monodon*. High levels of prostaglandins in polychaetes are believed to enhance shrimp ovarian development. However, the impact of polychaete feeding on shrimp prostaglandin biosynthesis and fatty acid regulatory pathways have yet to be investigated. As polychaetes contain higher levels of arachidonic acid (ARA), eicosapentaenoic acid (EPA), prostaglandin E₂ (PGE₂) and prostaglandin F_{2α} (PGF_{2α}) than feed pellets, we examined the effects of polychaete feeding alone and in combination with eyestalk ablation on shrimp hepatopancreases and ovaries. Shrimp fed with polychaetes contained higher levels of EPA, PGE₂ and PGF_{2α} in hepatopancreases than those of pellet-fed shrimp. Similarly, higher levels of ARA and higher transcription levels of cyclooxygenase (COX) and prostaglandin F synthase (PGFS) were detected in ovaries of polychaete-fed shrimp compared to those of pellet-fed shrimp. The combination of polychaete-feeding and eyestalk ablation, commonly practiced to induce ovarian development, increased levels of ARA and EPA and transcription levels of COX in hepatopancreases and ovaries of polychaete-fed shrimp compared to those of pellet-fed shrimp. In ovaries, prostaglandin biosynthesis gene transcripts were induced by polychaete feeding while transcriptional levels of fatty acid regulatory genes were regulated by shrimp feed and eyestalk ablation. Our findings not only elucidate the effects of polychaete consumption on shrimp prostaglandin biosynthesis and fatty acid regulatory pathways during larvae production, but also suggests that high levels of dietary ARA, EPA and prostaglandins are essential during *P. monodon* ovarian development.

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Introduction

Domestication of the black tiger shrimp *Penaeus monodon* has always been hindered by low ovarian maturation rates in female broodstock (Benzie 1997). Prostaglandin levels are thought to be significant factors, with their roles in crustacean ovarian maturation being investigated in several species. The amount of prostaglandin E₂ (PGE₂) in ovaries has been observed to increase during ovary development in the Florida crayfish *Procambarus paeninsulanus*, the freshwater field crab *Oziotelphusa senex senex* and *P. monodon* (Spaziani *et al.* 1993, Reddy *et al.* 2004, Meunpol *et al.* 2010, Wimuttisuk *et al.* 2013). In contrast, PGE₂ amounts decrease as oocytes mature in the freshwater prawn *Macrobrachium rosenbergii* and the kuruma prawn *Marsupenaeus japonicus* (Tahara & Yano 2004, Sumpownnon *et al.* 2015). The correlation between prostaglandin levels and oocyte maturation have been

demonstrated *in vitro*, with incubation of *P. monodon* oocytes in cell culture media spiked with synthetic PGE₂ or prostaglandin-rich polychaete extracts increasing the numbers of vitellogenic and mature oocytes (Meunpol *et al.* 2010). Similarly, injections of PGE₂ or prostaglandin F_{2α} (PGF_{2α}) in *O. senex senex* and PGE₂ in *M. rosenbergii* increased the gonadosomatic index (GSI) values in these crustaceans (Reddy *et al.* 2004, Sumpownnon *et al.* 2015).

Although prostaglandins have been shown to improve crustacean ovarian development, these prostaglandins were administered artificially. In hatcheries and shrimp farms, prostaglandins can be administered through feeding with polychaetes, with the ingested nutrients being stored and metabolized in hepatopancreases (Vogt 1994, Wen *et al.* 2001). Use of the polychaete *Perinereis nuntia* as shrimp feed is preferred as it contains the highest amount of PGE₂ among various live feeds, including mud polychaetes, horse mussels, squids and clams (Meunpol *et al.* 2010). Polychaetes

also contain higher amounts of arachidonic acid (ARA), a lipid precursor of prostaglandins, than other live feed (Meunpol *et al.* 2010, Chimsung 2014). Polychaete consumption has been shown to increase the amounts of ARA in hepatopancreases of *M. japonicus* (Binh *et al.* 2008). Increasing lipid content in crustacean hepatopancreases correlated with transcriptional changes in the fatty acid regulatory pathway, including beta-oxidation, metabolism, synthesis and transport in the Chinese mitten crab *Eriocheir sinensis*, the swimming crab *Portunus trituberculatus*, the Pacific white shrimp *Penaeus vannamei* and *P. monodon* (Chen *et al.* 2015, Wei *et al.* 2017, Deenarn *et al.* 2018, Yuan *et al.* 2019).

In this study, comparative analysis was performed to examine changes in the prostaglandin biosynthesis and fatty acid regulatory pathways due to shrimp feed. These changes were monitored in intact shrimp and after eyestalk ablation was performed, which resulted in shrimp with different stages of ovarian development. Together, our work provides new insights into metabolic and transcriptional changes that occur in female broodstock in the context of hatcheries and shrimp farms.

Materials and methods

Ethical statement

Shrimp trials and experiments in this study were approved by the Institutional Animal Care and Use Committee of the National Center for Genetic Engineering and Biotechnology, Thailand. All experiments were performed in accordance with Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) and conformed with international and national legal and ethical requirements (Elwood *et al.* 2009, Kilkenny *et al.* 2010). Shrimp were anesthetized by hypothermia to avoid animal suffering before dissections were performed.

Shrimp rearing and feed experiment

Eleven-month-old female domesticated *P. monodon* with an average body weight of 65.8 ± 14.0 g was transferred from earthen ponds and acclimated in six concrete tanks ($n=32$ shrimp per tank) for 11 days before the experiment began. Shrimp density was maintained at 2.5 shrimp/m². Shrimp were fed with B2 commercial feed pellets (Charoen Pokphand Food Public Company, Ltd., Bangkok, Thailand) and maintained in seawater at 30 ppt and 27°C. Ammonium, nitrite and nitrate levels, alkalinity and pH were monitored and adjusted twice a week as previously described (Deenarn *et al.* 2018).

Shrimp in three concrete tanks were fed with B2 commercial feed pellets and the rest was fed with fully mature *P. nuntia* at 2–10% of the shrimp body weight for 4 weeks, depending on the consumption rate. Intact shrimp were collected at week 0 (W0, $n=3$ per tank) and at the end of week 4 (W4, $n=3$ per tank). Unilateral eyestalk ablation was performed at the end of week 4 and broodstocks were maintained for 9 days. Sample collection occurred on days 1, 4, 7 and 9 (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of

this article). Shrimp body weights and lengths were measured and recorded. Hepatopancreases and ovaries were dissected, weighed and snap-frozen in liquid N₂.

The GSI of each broodstock was calculated using the following equation:

$$\text{GSI} = \frac{\text{ovary weight}}{\text{body weight}} \times 100$$

The ovarian developmental stage of each broodstock was assigned based on the GSI value: stage 1 (GSI < 2), stage 2 (GSI = 2–3.99), stage 3 (GSI = 4–6) and stage 4 (GSI > 6). The hepatosomatic index (HSI) was calculated using the following equation:

$$\text{HSI} = \frac{\text{hepatopancreas weight}}{\text{body weight}} \times 100$$

Nutritional analysis of shrimp feed

Fifty grams of feed pellets and polychaetes were sampled daily. Feed samples were pooled in a weekly batch and submitted for approximation and fatty acid profile analysis using gas chromatography-flame ionization detector (GC-FID) at the Central Laboratory (Thailand) Co., Ltd (Songkla, Thailand). Approximation was performed according to the Association of Official Agricultural Chemists (AOAC) guidelines. GC analyses were carried out using Agilent 6890N GC-FID (Agilent) equipped with a capillary column (100 m × 0.25 mm i.d., 0.2 µm, Supelco, Pennsylvania, USA). Detection and injection temperatures were set at 250°C. The flow rate of carrier gas (He) was set at 1.1 mL/min. Samples were compared with the standard fatty acid Supelco 37 component fatty acid methyl ester mixture using the in-house method TE-CH-208 based on AOAC guideline 996.06 (DeVries *et al.* 1999).

Extraction of polyunsaturated fatty acids (PUFAs) from shrimp tissues

Shrimp ovaries and hepatopancreases were homogenized and adjusted to 0.2 g tissue/mL in Hank's balanced salt solution (HBSS) at 720 mmol/kg osmolarity (Sigma-Aldrich). Tissue homogenates were extracted twice with an equal volume of ethyl acetate. The solvent phases were pooled, dried under vacuum and dissolved in 5% methanol. The extracts were loaded onto 6-mL C18 SPE cartridges (VertiPakTM, Vertical Chromatography, Co., Ltd., Thailand), which were pre-washed with 10 mL of methanol and 10 mL of water. Columns were then washed with 10 mL water, 4 mL hexane, and again with 10 mL water. Samples were eluted using 10 mL of ethyl acetate. The eluate was dried under vacuum and dissolved in ethanol for the reverse-phase HPLC (RP-HPLC) analysis.

Lipid composition analysis of organ extracts using RP-HPLC

Organ extracts were analyzed using RP-HPLC on an Acclaim® 120 C18 column (3 µm, 4.6 mm × 150 mm) (DIONEX, Surrey, UK).

The mobile phase consisted of 0.01% acetic acid in water (solvent A) and 0.01% acetic acid in acetonitrile (solvent B). The gradient program started with 30% solvent B and increased to 100% solvent B within 35 min. ARA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were detected using the DIONEX ultimate 3000 diode-array detector (DIONEX) and the amounts of ARA, EPA and DHA were calculated using the standard curve method as previously described (Wimuttisuk *et al.* 2013, 2017).

Enzyme immunoassay

Polychaetes, feed pellets, shrimp ovaries and hepatopancreases were homogenized in HBSS. Homogenates were centrifuged at 12,000g for 2 min at 4°C to remove cell debris. Amounts of PGE₂ and PGF_{2α} in the supernatant were estimated using the PGE₂ and PGF_{2α} enzyme immunoassay (EIA) kits (Cayman Chemical), respectively. Dose-response curves were generated with PGE₂ and PGF_{2α} standards at concentrations ranging from 15.63–250.00 pg/mL to 7.80–250.00 pg/mL, respectively. Limits of quantification were 15.63 pg/mL and 7.80 pg/mL for PGE₂ and PGF_{2α}, respectively. The average intra-day coefficient of variance (CV) of all controls was 6.41% for PGE₂ and 7.69% for PGF_{2α}. The average inter-day CV of all controls was 7.57% for PGE₂ and 10.50% for PGF_{2α}. For the PGE₂ EIA kit, percent cross reactivity was 0.07% for PGF_{2α} and less than 0.001% for prostaglandin D₂ (PGD₂) and ARA. For the PGF_{2α} EIA kit, percent cross reactivity was 0.66% for PGE₂ and less than 0.001% for PGD₂ and ARA.

RNA extraction and cDNA synthesis

Hepatopancreases and ovaries were subjected to total RNA extraction using the Trizol reagent (Invitrogen) and DNase treatment using RNase-free DNase (Promega). Total RNA was reverse transcribed using the RevertAid™ first strand cDNA Synthesis Kit with oligo (dT)₁₈ primers (Thermo Scientific).

Quantitative real-time PCR analysis

Quantitative real-time PCR analysis (qPCR) was performed on individual cDNA samples using the SsoFast EvaGreen Supermix (Bio-Rad) according to the manufacturer's instructions. Primer sequences for prostaglandin biosynthesis and fatty acid regulatory genes are provided in Supplementary Table 1. Melt curve analysis for each primer pair was performed as previously described (Deenarn *et al.* 2018). Amounts of target gene transcripts relative to that of elongation factor 1α (EF1A) were obtained using the standard curve method. Nine biological replicates were used for each time point and ovarian maturation stage (*n* = 9), except for the eyestalk-ablated, pellet-fed shrimp with stage 2 ovaries (*n* = 5).

Statistical analyses

Significant differences between the means of independent samples from the two feed groups were assessed using the *t*-test with the threshold for significance set at *P* < 0.05 (*) or *P* < 0.01 (**). Other statistical analyses were performed

using Dunnett's test to determine the significance between mean differences of designated samples. The thresholds for significance of the Dunnett's test were set at *P* < 0.05 (*, † and §) and *P* < 0.01 (*, †† and §§).

Results

Nutritional analysis of shrimp feed

Approximation analysis of shrimp feed revealed that polychaetes contained greater amounts of fat but less ash than feed pellets (Fig. 1A). Moisture, protein and fiber levels were comparable between the two feed types. GC-FID analysis revealed that feed pellets contained higher levels of six saturated fatty acids (C12:0, C14:0, C20:0, C22:0, C23:0 and C24:0), two monounsaturated fatty acids (C22:1n9 and C24:1n9) and three polyunsaturated fatty acids (C18:3n6, C20:3n3 and C22:6n3) than polychaetes (Supplementary Table 2). In contrast, polychaetes contained higher levels of 13 fatty acids, including ARA (C20:4n6) and EPA (C20:5n3), than feed pellets (Fig. 1B). The levels of PGE₂ and PGF_{2α}, which are products of ARA, were also higher in polychaetes than feed pellets (Fig. 1C).

Effects of shrimp feed on shrimp body weight, hepatosomatic index and ovarian development

Intact shrimp collected at W0 and W4 contained stage 1 ovaries regardless of feed type (Fig. 1D and E). After the eyestalk ablation was performed, pellet-fed shrimp reached stage 1 and 2 ovaries while polychaete-fed shrimp were able to reach all four ovarian maturation stages. Shrimp body weights were comparable between the polychaete- and pellet-fed groups at every time point (Fig. 1F). On the other hand, the color and size of hepatopancreases were different between shrimp fed with feed pellets and polychaetes (Fig. 1G). The average hepatosomatic index (HSI) of shrimp fed with polychaetes was also higher than those fed with feed pellets after 4 weeks of feeding and in eyestalk-ablated samples (Fig. 1H).

Changes in hepatopancreases of intact shrimp

To determine whether the amounts of ARA, EPA and DHA in shrimp hepatopancreases were affected by shrimp feed, RP-HPLC analysis was performed on hepatopancreases of intact shrimp collected at W0 and W4. At W4, levels of EPA in hepatopancreases of polychaete-fed shrimp were higher than those of pellet-fed shrimp (Fig. 2B). Additionally, levels of ARA, EPA and DHA in hepatopancreases of polychaete-fed shrimp at W4 were also higher than those at W0 (Fig. 2A, B and C). RT-qPCR analysis was also performed on these samples, revealing that hepatopancreases of polychaete-fed shrimp at W4 showed increased transcription levels

Figure 1 Nutritional assessment of shrimp feed and their effects on shrimp body weight, GSI and HSI. (A, B and C) Fifty grams of feed pellets and polychaetes were collected daily during the feed trial and pooled in a weekly group for analysis ($n=5$). (A) Nutritional analysis of feed pellets and polychaetes was performed to determine the amounts of fat, ash, moisture, protein and fiber. (B) Levels of ARA, EPA and DHA in shrimp feed were determined. (C) Levels of PGE₂ and PGF_{2α} in shrimp feed were determined using enzyme immunoassays. (D) The number of shrimp, (E) average GSI values and (F) average shrimp body weights were analyzed according to feed groups, time points and ovarian maturation stages. (G) Shrimp hepatopancreases collected from shrimp with stage 1 ovaries that were fed with feed pellets (top panel) and polychaetes (bottom panel) at week 0 (W0S1), after 4 weeks of feeding (W4S1) and after eyestalk ablation (W4EAS1). (H) Average HSI values of shrimp from different feed groups, time points and ovarian maturation stages. Error bars indicate standard deviations. Asterisks indicate significant differences between feed pellets and polychaetes at $P < 0.05$. #, ## Significant differences between the designated condition and the control at W0 at $P < 0.05$ and $P < 0.01$, respectively. \$\$ Significant differences between the marked condition and the pellet-fed shrimp at W4EAS1 at $P < 0.01$. ++ Significant differences between the marked condition and the polychaete-fed shrimp at W4EAS1 at $P < 0.01$. N/A indicates that the shrimp sample was not available in the marked stage. Different ovarian maturation stages were shown as S1 for stage 1, S2 for stage 2, S3 for stage 3 and S4 for stage 4.

of cytosolic phospholipase A2 (cPLA2) and prostaglandin F synthase (PGFS) but decreased transcription levels of cyclooxygenase (COX) when compared to those of pellet-fed shrimp at W4 (Fig. 2D, E and I). No significant difference was observed for genes involved in PGE₂ production, namely prostaglandin E synthase 1 (PGES1), prostaglandin E synthase 2 (PGES2) and prostaglandin E synthase 3 (PGES3) (Fig. 2F, G and H). However, levels of PGE₂ and PGF_{2α} in hepatopancreases of polychaete-fed shrimp were 15 and 12 times higher than those in pellet-fed shrimp, respectively (Fig. 2J and K).

Due to different fatty acid profiles in shrimp feed, changes in fatty acid regulatory gene expression were examined in shrimp hepatopancreases after 4 weeks of feeding. Hepatopancreases of polychaete-fed shrimp contained higher transcription levels of stearoyl-CoA desaturase (SCD) and lower transcriptional levels of enoyl-CoA hydratase (ECH) than those of pellet-fed shrimp (Fig. 3A and I). Additionally, transcription levels of two fatty acid transport genes, long-chain fatty acid transport protein 4 (FATP4) and fatty acid-binding protein (FABP), also increased as feeding time progressed

Figure 2 Effects of shrimp feed on the prostaglandin biosynthesis pathway in shrimp hepatopancreases. Shrimp hepatopancreases were collected at the beginning of the feed experiment when shrimp were only fed with feed pellets (W0, $n=18$) and again after shrimp were fed with either feed pellets (white bars, $n=9$) or polychaetes (black bars, $n=9$) for 4 weeks (W4). All shrimp samples contained stage 1 ovaries. Levels of (A) ARA, (B) EPA and (C) DHA in hepatopancreases were determined. Expression levels of (D) *cPLA2*, (E) *COX*, (F) *PGES1*, (G) *PGES2*, (H) *PGES3* and (I) *PGFS* in shrimp hepatopancreases were determined using RT-qPCR and expressed relative to *EF1A*. Levels of (J) PGE₂ and (K) PGF_{2α} in shrimp hepatopancreases were determined using enzyme immunoassays. Error bars indicate standard deviations. *,**Significant differences between feed pellets and polychaetes at $P < 0.05$ and $P < 0.01$, respectively. #,##Significant differences between the designated condition and the control at W0 at $P < 0.05$ and $P < 0.01$, respectively.

from W0 to W4 in hepatopancreases of polychaete-fed shrimp (Fig. 3E and F).

Effects of shrimp feed on ovaries of intact shrimp

Ovaries of polychaete-fed shrimp contained higher levels of ARA and lower levels of DHA than those of pellet-fed shrimp at W4. (Fig. 4A and C). Transcription levels of *COX*, *PGES3* and *PGFS* in ovaries of polychaete-fed shrimp were higher than those of pellet-fed shrimp (Fig. 4E, H and I). On the other hand, transcription levels of *cPLA2*, *PGES1* and *PGES2* and levels of PGE₂ and PGF_{2α} in ovaries were not affected by shrimp diet (Fig. 4D, F, G, J and K). Ovaries of polychaete-fed shrimp showed higher transcription levels of *SCD*, lipophorin receptor (*LPR*), long-chain fatty acid transport protein 1 (*FATP1*), *FATP4* and *ECH* than those of pellet-fed shrimp (Fig. 5A, C, D, E and I). In contrast, transcription levels of fatty acid synthase (*FASN*) in ovaries of polychaete-fed shrimp were lower than those of pellet-fed shrimp (Fig. 5B).

Combined effects of shrimp feed and eyestalk ablation on shrimp hepatopancreases

During shrimp larvae production, a combination of polychaete feeding and eyestalk ablation is required to induce successful ovarian development. Due to the lack of eyestalk-ablated, pellet-fed shrimp with stage 3 and 4 ovaries, the combined effects of shrimp feed and eyestalk ablation were determined only in shrimp with stage 1 and 2 ovaries. Hepatopancreases of polychaete-fed shrimp with stage 1 ovaries contained higher levels of ARA while those with stage 2 ovaries contained higher levels of EPA and DHA compared to those of pellet-fed shrimp (Fig. 6A, B and C). Higher transcription levels of *COX* and *PGES3*, but lower transcription levels of *PGES1* were detected in hepatopancreases of polychaete-fed shrimp with stage 1 ovaries compared to those of pellet-fed shrimp (Fig. 6E, F and H). When shrimp reached stage 2 ovaries, transcription levels of all six prostaglandin biosynthesis genes were comparable in hepatopancreases of shrimp from both feed groups

Figure 3 Effects of shrimp feed on relative expression levels of fatty acid regulatory genes in shrimp hepatopancreases. Shrimp hepatopancreases were collected at the beginning of the feed experiment when shrimp were only fed with feed pellets (W0, $n=18$) and again after shrimp were fed with either feed pellets (white bars, $n=9$) or polychaetes (black bars, $n=9$) for 4 weeks (W4). All shrimp samples contained stage 1 ovaries. Expression levels of fatty acid regulatory genes in shrimp hepatopancreases collected at W0 and W4 were determined using RT-qPCR and expressed relative to *EF1A*. Fatty acid regulatory genes include (A) *SCD* and (B) *FASN* in the fatty acid biosynthesis pathway; (C) *LPR*, (D) *FATP1*, (E) *FATP4* and (F) *FABP* in the fatty acid transport pathway; and (G) *ACAT*, (H) *ACAD* and (I) *ECH* in the fatty acid beta-oxidation pathway. Error bars indicate standard deviations. *,**Significant differences between feed pellets and polychaetes at $P < 0.05$ and $P < 0.01$, respectively. #,##Significant differences between the designated condition and the control at W0 at $P < 0.05$ and $P < 0.01$, respectively.

(Fig. 6D, E, F, G, H and I). Nevertheless, the levels of PGE_2 and $PGF_{2\alpha}$ in hepatopancreases of shrimp fed with polychaetes remained 15 and 12 times higher, respectively, than those fed with feed pellets (Fig. 6J and K). As prostaglandin levels in hepatopancreases of polychaete-fed shrimp were higher than those of pellet-fed shrimp in both intact and eyestalk-ablated samples, we speculate that the accumulation of prostaglandins in shrimp hepatopancreases were due to high levels of prostaglandins found in polychaetes. Eyestalk ablation did not affect the levels of prostaglandins in this organ.

The combination of shrimp feed and eyestalk ablation had minimal impact on fatty acid regulatory gene transcripts in shrimp hepatopancreases. In eyestalk-ablated shrimp with stage 2 ovaries, transcription levels of acyl-CoA dehydrogenase (*ACAD*) were higher in hepatopancreases of shrimp fed with polychaetes compared to those fed with feed pellets (Fig. 7H). The rest of the fatty acid regulatory genes showed similar transcription levels in hepatopancreases of shrimp in both feed groups (Fig. 7A, B, C, D, E, F, G and I).

Combined effects of shrimp feed and eyestalk ablation on shrimp ovaries

In eyestalk-ablated samples, levels of ARA and EPA were higher in ovaries of polychaete-fed shrimp compared to those of pellet-fed shrimp (Fig. 8A and B). Additionally,

polychaete-fed shrimp with stage 1 ovaries exhibited higher transcription levels of *COX* and *PGFS* than those fed with feed pellets (Fig. 8E and I). At stage 2, transcription levels of *PGES2* were higher in ovaries of polychaete-fed shrimp than those of pellet-fed shrimp (Fig. 8G). Enzyme immunoassays revealed that levels of PGE_2 were comparable in ovaries of both feed groups while levels of $PGF_{2\alpha}$ were higher in stage 1 ovaries of pellet-fed shrimp compared to those of polychaete-fed shrimp (Fig. 8J and K).

Although higher transcription levels of fatty acid regulatory genes were detected in ovaries of intact polychaete-fed shrimp than those of pellet-fed shrimp, the reverse correlations were observed in ovaries of eyestalk-ablated samples. Transcription levels of *FABP*, acetyl-CoA acetyltransferase (*ACAT*) and *ACAD* were higher in stage 1 ovaries of pellet-fed shrimp than those of polychaete-fed shrimp (Fig. 9F, G and H). As shrimp ovaries progressed to stage 2, transcription levels of *SCD*, *LPR* and *FABP* were higher in ovaries of pellet-fed shrimp compared to those of polychaete-fed shrimp (Fig. 9A, C and F).

Changes in hepatopancreases of polychaete-fed shrimp during ovarian maturation

Aside from comparing differences between the two feed groups, data analysis was performed to assess changes in

Figure 4 Effects of shrimp feed on prostaglandin biosynthesis pathway in shrimp ovaries. Shrimp ovaries were collected at the beginning of the feed experiment when shrimp were only fed with feed pellets (W0, $n=18$) and again after shrimp were fed with either feed pellets (white bars, $n=9$) or polychaetes (black bars, $n=9$) for 4 weeks (W4). All shrimp samples contained stage 1 ovaries. Levels of (A) ARA, (B) EPA and (C) DHA in ovaries were determined. Expression levels of (D) *cPLA2*, (E) *COX*, (F) *PGES1*, (G) *PGES2*, (H) *PGES3* and (I) *PGFS* in shrimp ovaries were determined using RT-qPCR and expressed relative to *EFTA*. Levels of (J) PGE₂ and (K) PGF_{2α} in shrimp ovaries were determined using enzyme immunoassays. Error bars indicate standard deviations. **, *Significant differences between feed pellets and polychaetes at $P < 0.05$ and $P < 0.01$, respectively. #, ##Significant differences between the designated condition and the control at W0 at $P < 0.05$ and $P < 0.01$, respectively.

the hepatopancreases of polychaete-fed shrimp during ovarian development. Compared to stage 1 ovaries, levels of DHA and EPA in hepatopancreases of polychaete-fed shrimp increased as shrimp reached stage 2 and 3 ovaries, respectively (Fig. 6B and C). Transcription levels of *PGES1* in hepatopancreases were also highest in shrimp with stage 2 ovaries (Fig. 6F). In contrast, transcription levels of *cPLA2*, *COX*, *PGES2*, *PGES3* and *PGFS* decreased as ovaries matured (Fig. 6D, E, G, H and I). Levels of PGE₂ and PGF_{2α} in the hepatopancreases of polychaete-fed shrimp remained unchanged throughout ovarian development (Fig. 6J and K). Additionally, transcription levels of *FASN*, *LPR*, *FABP* and *ACAD* decreased while transcription levels of *SCD* and *FATP1* increased in shrimp hepatopancreases during ovarian development (Fig. 7A, B, C, D, F and H).

Changes in ovaries of polychaete-fed shrimp during ovarian maturation

In ovaries of eyestalk-ablated, polychaete-fed shrimp, levels of ARA and EPA were highest in shrimp with

stage 1 ovaries and decreased as ovaries matured (Fig. 8A and B). Transcription levels of *cPLA2*, *PGES1*, *PGES2*, and *PGES3* increased as ovaries progressed from stage 1 to 4 (Fig. 8D, F, G and H). In contrast, levels of PGE₂ in ovaries decreased while levels of PGF_{2α} remained unchanged during ovarian development (Fig. 8J and K). Transcription levels of seven fatty acid regulatory genes, namely *SCD*, *FASN*, *FATP1*, *FABP*, *ACAT*, *ACAD* and *ECH*, increased while transcription levels of *LPR* and *FATP4* decreased in ovaries during ovarian development (Fig. 9).

Discussion

Several nutritional factors, including proteins, PUFAs, carbohydrates and vitamins, contribute to successful ovarian development in crustaceans (Castille & Lawrence 1989, Xu et al. 1994, Cahu et al. 1995, Wouters et al. 2001). Previous studies propose that polychaetes contain higher levels of signaling molecules and hormones, such as prostaglandins and

Figure 5 Effects of shrimp feed on relative expression levels of fatty acid regulatory genes in shrimp ovaries. Shrimp ovaries were collected at the beginning of the feed experiment when shrimp were only fed with feed pellets (W0, $n=18$) and again after shrimp were fed with either feed pellets (white bars, $n=9$) or polychaetes (black bars, $n=9$) for 4 weeks (W4). All shrimp samples contained stage 1 ovaries. Expression levels of fatty acid regulatory genes in shrimp ovaries collected at W0 and W4 were determined using RT-qPCR and expressed relative to *EF1A*. Fatty acid regulatory genes include (A) *SCD* and (B) *FASN* in the fatty acid biosynthesis pathway; (C) *LPR*, (D) *FATP1*, (E) *FATP4* and (F) *FABP* in the fatty acid transport pathway; and (G) *ACAT*, (H) *ACAD* and (I) *ECH* in the fatty acid beta-oxidation pathway. Error bars indicate standard deviations. * **Significant differences between feed pellets and polychaetes at $P < 0.05$ and $P < 0.01$, respectively. #, ##Significant differences between the designated condition and the control at W0 at $P < 0.05$ and $P < 0.01$, respectively.

Figure 6 Combined effects of shrimp feed and eyestalk ablation on prostaglandin biosynthesis pathway in shrimp hepatopancreases. Eyestalk-ablated shrimp that had previously been fed with feed pellets (white bars) or polychaetes (black bars) were grouped based on ovarian maturation stages (S1 for stage 1, S2 for stage 2, S3 for stage 3 and S4 for stage 4). Levels of (A) ARA, (B) EPA and (C) DHA in hepatopancreases were determined. Expression levels of (D) *cPLA2*, (E) *COX*, (F) *PGES1*, (G) *PGES2*, (H) *PGES3* and (I) *PGFS* in shrimp hepatopancreases were determined using RT-qPCR and expressed relative to *EF1A*. Levels of (J) *PGE2* and (K) *PGF2α* in shrimp hepatopancreases were determined using enzyme immunoassays. Error bars indicate standard deviations. * **Significant differences between feed pellets and polychaetes at $P < 0.05$ and $P < 0.01$, respectively. #, ##Significant differences between the marked condition and the pellet-fed shrimp at W4EAS1 at $P < 0.05$ and $P < 0.01$, respectively. †, ‡Significant differences between the marked condition and the polychaete-fed shrimp at W4EAS1 at $P < 0.05$ and $P < 0.01$, respectively. §, §§Significant differences between the marked condition and the polychaete-fed shrimp at W4EAS1 at $P < 0.05$ and $P < 0.01$, respectively. N/A indicates that shrimp samples were not available for that stage.

Figure 7 Combined effects of shrimp feed and eyestalk ablation on fatty acid regulatory genes in shrimp hepatopancreases. Eyestalk-ablated shrimp that had previously been fed with feed pellets (white bars) or polychaetes (black bars) were grouped based on ovarian maturation stages (S1 for stage 1, S2 for stage 2, S3 for stage 3 and S4 for stage 4). Expression levels of fatty acid regulatory genes in ovaries of eyestalk-ablated shrimp were determined using RT-qPCR and expressed relative to *EF1A*. Fatty acid regulatory genes include (A) *SCD* and (B) *FASN* in the fatty acid biosynthesis pathway; (C) *LPR*, (D) *FATP1*, (E) *FATP4* and (F) *FABP* in the fatty acid transport pathway; and (G) *ACAT*, (H) *ACAD* and (I) *ECH* in the fatty acid beta-oxidation pathway. Error bars indicate standard deviations. *, **Significant differences between feed pellets and polychaetes at $P < 0.05$ and $P < 0.01$, respectively. \$, \$\$ indicate significant differences between the marked condition and the pellet-fed shrimp at W4EAS1 at $P < 0.05$ and $P < 0.01$, respectively. †, ††Significant differences between the marked condition and the polychaete-fed shrimp at W4EAS1 at $P < 0.05$ and $P < 0.01$, respectively. N/A indicates that shrimp samples were not available for that stage.

Figure 8 Combined effects of shrimp feed and eyestalk ablation on prostaglandin biosynthesis in shrimp ovaries. Eyestalk-ablated shrimp that had previously been fed with feed pellets (white bars) or polychaetes (black bars) were grouped based on ovarian maturation stages (S1 for stage 1, S2 for stage 2, S3 for stage 3 and S4 for stage 4). Levels of (A) ARA, (B) EPA and (C) DHA in ovaries were determined. Expression levels of (D) *cPLA2*, (E) *COX*, (F) *PGES1*, (G) *PGES2*, (H) *PGES3* and (I) *PGFS* in shrimp ovaries were determined using RT-qPCR and expressed relative to *EF1A*. Levels of (J) *PGE₂* and (K) *PGF_{2α}* in shrimp ovaries were determined using enzyme immunoassays. Error bars indicate standard deviations. *, **Significant differences between feed pellets and polychaetes at $P < 0.05$ and $P < 0.01$, respectively. \$, \$\$Significant differences between the marked condition and the pellet-fed shrimp at W4EAS1 at $P < 0.05$ and $P < 0.01$, respectively. †, ††Significant differences between the marked condition and the polychaete-fed shrimp at W4EAS1 at $P < 0.05$ and $P < 0.01$, respectively. N/A indicates that shrimp samples were not available for that stage.

Figure 9 Combined effects of shrimp feed and eyestalk ablation on fatty acid regulatory genes in shrimp ovaries. Eyestalk-ablated shrimp that had previously been fed with feed pellets (white bars) or polychaetes (black bars) were grouped based on ovarian maturation stages (S1 for stage 1, S2 for stage 2, S3 for stage 3 and S4 for stage 4). Expression levels of fatty acid regulatory genes in ovaries of eyestalk-ablated shrimp were determined using RT-qPCR and expressed relative to *EF1A*. Fatty acid regulatory genes include (A) *SCD* and (B) *FASN* in the fatty acid biosynthesis pathway; (C) *LPR*, (D) *FATP1*, (E) *FATP4* and (F) *FABP* in the fatty acid transport pathway; and (G) *ACAT*, (H) *ACAD* and (I) *ECH* in the fatty acid beta-oxidation pathway. Error bars indicate standard deviations. *Significant differences between feed pellets and polychaetes at $P < 0.05$ and $P < 0.01$, respectively. \$, \$\$Significant differences between the marked condition and the pellet-fed shrimp at W4EAS1 at $P < 0.05$ and $P < 0.01$, respectively. †, ††Significant differences between the marked condition and the polychaete-fed shrimp at W4EAS1 at $P < 0.05$ and $P < 0.01$, respectively. N/A indicates that shrimp samples were not available for that stage.

progesterone, which are responsible for successful ovarian development in crustaceans (Reddy *et al.* 2004, Meunpol *et al.* 2007, 2010). In this study, changes in the prostaglandin biosynthesis pathway of shrimp fed with feed pellets and polychaetes were examined as the effects of shrimp feed alone or in combination with eyestalk ablation. Additionally, transcriptional changes of fatty acid regulatory genes were examined to determine the connection between shrimp diet, prostaglandins and fatty acid regulatory pathways in *P. monodon*.

Polychaete feeding and the levels of PUFAs and prostaglandins in crustaceans

A study in *M. japonicus* showed that hepatopancreases of polychaete-fed shrimp contained larger amounts of ARA than those of pellet-fed shrimp (Binh *et al.* 2008). In male *P. monodon*, consumption of natural diets comprised of polychaetes, squid and mussels also resulted in larger amounts of ARA in shrimp hepatopancreases when compared to those fed with commercial feed pellets (Meunpol *et al.* 2005). Similarly, our study showed that ovaries of shrimp fed with polychaetes showed higher levels of ARA and higher transcription levels of *COX* and *PGFS* than those of pellet-fed shrimp. However, the levels of PGE_2 and $\text{PGF}_{2\alpha}$ in ovaries did not share the same correlation. We speculate that PGE_2 and $\text{PGF}_{2\alpha}$ were quickly synthesized and then converted to downstream metabolites by enzymes such as prostaglandin reductase 1 (Prasertlux *et al.* 2011). Therefore, the levels of these

prostaglandins did not change significantly when detected with enzyme immunoassays.

In hepatopancreases, high levels of ARA and EPA in polychaetes resulted in higher levels of ARA and EPA in hepatopancreases of eyestalk-ablated, polychaete-fed shrimp compared to those of pellet-fed shrimp. This corresponded with increased transcription levels of *COX* and *PGES3* in shrimp hepatopancreases. Additionally, high levels of PGE_2 and $\text{PGF}_{2\alpha}$ in polychaetes also correlated with higher levels of PGE_2 and $\text{PGF}_{2\alpha}$ in hepatopancreases of polychaete-fed shrimp compared to those of pellet-fed shrimp.

PUFAs, prostaglandins and ovarian development

As ovarian maturation progressed, the amounts of ARA and EPA decreased while the amount of DHA increased in the banana shrimp *Penaeus merguensis* (Chansela *et al.* 2012). Reduction of ARA and EPA levels was also observed as *P. monodon* ovaries matured. On the other hand, different levels of prostaglandins in ovaries have been shown to correlate with ovarian development in crustaceans, including *P. monodon*, *M. rosenbergii*, *P. paeninsulanus*, *M. japonicus* and *O. senex senex* (Spaziani *et al.* 1993, Reddy *et al.* 2004, Tahara & Yano 2004, Meunpol *et al.* 2010, Wimuttisuk *et al.* 2013, Sumpownon *et al.* 2015). Levels of PGE_2 in ovaries decreased in *M. japonicus* and *M. rosenbergii*, but increased in *P. paeninsulanus*, *O. senex senex* and wild *P. monodon* during ovarian development

(Spaziani *et al.* 1993, Reddy *et al.* 2004, Tahara & Yano 2004, Meunpol *et al.* 2010, Wimuttisuk *et al.* 2013, Sumpownon *et al.* 2015). On the other hand, levels of PGE₂ in domesticated *P. monodon* ovaries decreased as ovaries matured, suggesting opposing correlations between wild and domesticated shrimp. Therefore, it is unlikely that the level of PGE₂ in ovaries is the determining factor for successful ovarian development.

Changes in the transcription levels of prostaglandin biosynthesis genes have previously been reported in two studies. In the female mud crab *Scylla olivacea*, *PGES* levels significantly increased from stage 1 to stage 4 ovaries (Duangprom *et al.* 2018). In wild *P. monodon*, transcription levels of *PGES1* increased while transcription levels of *PGES2* and *PGFS* decreased in shrimp ovaries during ovarian development (Wimuttisuk *et al.* 2013). In this study, transcription levels of prostaglandin biosynthesis genes in ovaries of polychaete-fed shrimp increased, while levels of PGE₂ decreased as ovarian maturation progressed. Although this suggests opposing correlations at the transcription and metabolic levels, the decreasing levels of PGE₂ corresponded with decreasing levels of ARA in shrimp ovaries. Therefore, the levels of prostaglandins were likely regulated by the availability of the precursor ARA rather than the levels of prostaglandin gene transcripts.

Effects of shrimp diet and ovarian maturation on fatty acid regulatory genes

Changes in crustacean diet have previously been shown to affect transcription levels of fatty acid regulatory genes, including *FABP*, *FASN* and *SCD* in *E. sinensis* and *P. vannamei* (Wen *et al.* 2001, Guo *et al.* 2013, Chen *et al.* 2015, Wei *et al.* 2017). In *P. monodon*, transcription levels of *SCD*, *LPR*, *FATP1*, *FATP4* and *ECH* increased while transcription levels of *FASN* decreased in ovaries of polychaete-fed shrimp compared to those of pellet-fed shrimp. Both *SCD* and *FASN* are part of the fatty acid synthesis pathway, in which *SCD* regulates the rate-limiting step of monounsaturated fatty acids synthesis while *FASN* is crucial for long-chain fatty acid synthesis (Enoch *et al.* 1976, Chakravarty *et al.* 2004). Studies have shown that *SCD* mRNA stability is regulated by dietary PUFAs and cholesterol while transcription levels of *FASN* were found to be reduced in hamsters on a high fat diet (Ntambi 1999, Field *et al.* 2003). Therefore, the impact of shrimp diet on *SCD* and *FASN* transcripts indicates that this regulatory mechanism is also conserved in crustaceans.

During *P. monodon* ovarian development, transcription levels of *ACAT*, *ACAD* and *ECH* increased in shrimp ovaries. These genes are part of the fatty acid beta-oxidation pathway, which metabolizes fatty acids for ATP production (Houten & Wanders 2010). Our data

Figure 10 Summary of the effects of shrimp feed on prostaglandin biosynthesis and fatty acid regulatory pathways in *P. monodon*. The left panel illustrates the effects of shrimp feed on intact shrimp. The middle panel represents the combined effects of shrimp feed and eyestalk ablation on shrimp hepatopancreases and ovaries. Changes in polychaete-fed shrimp during ovarian development are shown in the right panel. Black arrows indicate higher levels of PUFAs and prostaglandins compared to the other feed group. White arrows indicate higher transcription levels of the designated gene compared to those in shrimp from the other feed group. Gray arrows point up or down indicate that the levels of PUFAs and PGE₂ in the designated organ increased or decreased, respectively, as ovaries matured. Red and green arrows represent higher and lower transcription levels of genes as ovarian maturation progressed compared to eyestalk-ablated, polychaete-fed shrimp with stage 1 ovaries, respectively.

correspond with results from other studies that fatty acid beta-oxidation is up-regulated in ovaries during ovarian development due to increased energy requirements (Paczkowski *et al.* 2013, Dunning *et al.* 2014, Deenarn *et al.* 2018).

Dynamics of prostaglandin biosynthesis and fatty acid regulatory pathways in *P. monodon*

The effects of polychaete consumption on shrimp prostaglandin biosynthesis and fatty acid regulatory pathways in this study can be categorized into three stages: (1) in intact shrimp after 4 weeks of feeding, (2) in eyestalk-ablated shrimp, and (3) changes that occurred in polychaete-fed shrimp during ovarian development (Fig. 10). Intact shrimp fed with polychaetes contained higher levels of EPA, PGE₂ and PGF_{2α} in their hepatopancreases than pellet-fed shrimp. However, the difference in shrimp feed had minor effects on the transcription levels of prostaglandin biosynthesis and fatty acid regulatory genes in shrimp hepatopancreases. On the other hand, ovaries of polychaete-fed shrimp contained higher levels of ARA but lower levels of DHA than those of pellet-fed shrimp. The transcription levels of prostaglandin biosynthesis and fatty acid regulatory genes generally increased in ovaries of polychaete-fed shrimp compared to those of pellet-fed shrimp.

After eyestalk ablation was performed, levels of ARA, EPA, DHA, PGE₂ and PGF_{2α} in hepatopancreases of polychaete-fed shrimp were higher than those fed with feed pellets. Most prostaglandin biosynthesis and fatty acid regulatory genes in hepatopancreases were unaffected by the difference in shrimp feed. Ovaries of eyestalk-ablated, polychaete-fed shrimp contained higher levels of ARA and EPA, but lower levels of PGF_{2α} than those of pellet-fed shrimp. Prostaglandin biosynthesis and fatty acid regulatory genes in ovaries of eyestalk-ablated shrimp showed opposing transcriptional patterns, with transcription levels of prostaglandin biosynthesis genes being higher in ovaries of polychaete-fed shrimp and transcription levels of fatty acid regulatory genes being higher in ovaries of pellet-fed shrimp.

Lastly, transcription and metabolic changes were monitored in polychaete-fed shrimp as ovary development progressed from stage 1 to stage 4. In hepatopancreases, levels of EPA and DHA increased while transcriptional levels of prostaglandin biosynthesis and fatty acid regulatory genes decreased as ovarian maturation progressed. In shrimp ovaries, however, levels of ARA, EPA and PGE₂ decreased but transcription levels of most prostaglandin biosynthesis and fatty acid regulatory genes increased as ovaries matured. Together, our results indicate that polychaete feeding induced the prostaglandin biosynthesis pathway in *P. monodon*. The dynamics between prostaglandin biosynthesis and fatty acid regulatory pathways in shrimp ovaries and hepatopancreas were observed at various stages during

ovarian maturation, providing insight into the transcription and metabolic changes that occur in crustaceans before and during ovarian development. Lastly, our data suggests a new strategy for feed formulation, in which higher amounts of ARA, EPA, PGE₂ and PGF_{2α} in feed pellets are imperative to induce successful ovarian development.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0217>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

P D and P T performed experiments and analyzed data. V V and R L planned experiments and revised the paper. S P and P C conducted shrimp feed trials. W W planned and performed experiments, analyzed data and wrote the paper.

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