Nutritional programming of Pacific whiteleg shrimp *Litopaneus vannamei*

**Programmation nutritionnelle de la crevette du Pacifique à pattes blanches *Litopaneus vannamei***

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS IV
LIST OF FIGURES VI
LIST OF TABLES VIII

Chapter 1 Introduction 2
  1.1 Aquaculture and shrimp production 3
    1.1.1 History of aquaculture and with emphasis on shrimp farming 3
    1.1.2 Shrimp Farming 5
  1.2 The biology of *Litopenaeus vannamei* 11
    1.2.1 The cycle, ontogenesis, feeding habits, morphological characterization and growth pattern of *L. vannamei* 11
    1.2.2 Life cycle and feeding differentiation 15
  1.3 Shrimp Nutrition 17
    1.3.1 Diets for farmed shrimp and nutritional requirement 17
      1.3.1.1 Protein and amino acid requirement 19
      1.3.1.2 Lipids requirement 21
      1.3.1.2 Carbohydrate requirements 23
    1.3.2 Development of novel diets for shrimp aquaculture 25
  1.4 Use of nutrients: digestion process and intermediary metabolism 29
    1.4.1 Digestion and juvenile digestion anatomy 29
    1.4.2 Intermediary metabolism 31
      1.4.2.1 Amino acids metabolism 33
      1.4.2.2 Glucose metabolism 35
      1.4.2.3 Lipid metabolism 43
      1.4.2.4 Energy (mitochondrial) metabolism 45
  1.5 Nutritional Programming – searching for new feeding strategies 47
    1.5.1 Concept of Nutritional programming 47
    1.5.2 Mechanisms of epigenetics: focus on aquaculture species 53
      1.5.2.1 DNA methylation and gene expression 55
      1.5.2.2 Main mechanisms involved in DNA methylation 55

Chapter 2 Thesis objectives 58

Chapter 3 Material and methods 60
  3.1 *The ontogenic study* 61
    3.1.1 Life cycle identification and sampling 61
    3.1.2 Primers design for analysis of mRNA levels 63
    3.1.3 RNA extraction 63
    3.1.4 Quantification of target genes by qRT-PCR 67
  3.2 *Characterization of experimental units for the programming studies* 69
  3.3 *Nutritional programming study at the protozoea phase* 71
    3.3.1 Experimental design 73
    3.3.2 Stimulus protocol through feed restriction 73
    3.3.3 Samplings 75
  3.4 *Nutritional programming at the post-larvae phase* 75
    3.4.1 Experimental design 75
    3.4.2 Stimulus and hatchery 77
    3.4.3 Diet production 77
    3.4.4 Dietary challenge for juvenile shrimps 83
    3.4.5 Samplings 83
      3.4.5.1 Hemolymph and plasma sampling 85
      3.4.5.2 Larvae samples (whole body sampling) for total RNA extraction 85
      3.4.5.3 Tissue sampling for total RNA extraction 85
3.4.6 Metabolites analyses 85
3.4.7 DNA extraction 87
3.4.8 Global DNA methylation evaluation 87
3.5 Statistical analyzes 87

Chapter 4 Results 88
Publication 1 90
Publication 2 100
Supplementary data 114
Publication 3 116

Chapter 5 General discussion 137
5.1 The choice of two developmental windows (protozoa and post-larvae) to perform the nutritional programming in whiteleg shrimp 139
5.2 The early feed restriction stimulus was performed at two developmental stages in whiteleg shrimp 144
5.3 Was it possible to program the whiteleg shrimp through an early nutritional stimulus 147
5.3.1 It was possible to program L. vannamei juveniles after an early feed stimulus (feed restriction) at the post-larvae stage. 147
5.3.2 A moderate feed restriction stimulus at early protozoa stage did not strongly modify in the long-term the growth performance, digestion and metabolism of L. vannamei. 151
5.4 The decrease of the dietary proteins/carbohydrates ratios in juveniles of whiteleg shrimps: consequences on growth performances and molecular hepatic metabolism. 153
5.5 Could the use of the different diets be modified by the early feed restriction stimulus? 157

Chapter 6 Conclusions and perspectives 158
Conclusions 158
Perspectives 161
6.1.1.1 Further research for new stimuli 161
6.1.1.2 Evaluation of new developmental windows to perform the stimuli 162
6.1.1.3 Research of new biological targets in order to test the programming concept 162

BIBLIOGRAPHY 163
LIST OF PUBLICATIONS 185
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆9/SCD</td>
<td>∆9-desaturase (stearoyl-CoA desaturase, SCD)</td>
</tr>
<tr>
<td>5hmC</td>
<td>5-hydroxymethylcytosine</td>
</tr>
<tr>
<td>5mC</td>
<td>5-methylcytosine</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AFI</td>
<td>Apparent feed intake</td>
</tr>
<tr>
<td>ANF</td>
<td>Anti-nutritional factors</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>atpase a</td>
<td><em>Litopenaeus vannamei</em> mitochondrial atp synthase subunit alpha precursor, mRNA</td>
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<tr>
<td>atpase b</td>
<td><em>Litopenaeus vannamei</em> mitochondrial atp synthase subunit beta precursor, mRNA</td>
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<tr>
<td>BUN</td>
<td>nitrogen excretions through gill and urine</td>
</tr>
<tr>
<td>CBH</td>
<td>Carbohydrate</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>CH₃</td>
<td>methyl group</td>
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<tr>
<td>chymotrypsin</td>
<td>chymotrypsin b II (<em>Litopenaeus vannamei</em>)</td>
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<tr>
<td>chh</td>
<td><em>Litopenaeus vannamei</em> crustacean hyperglycemic hormone</td>
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<tr>
<td>-COOH</td>
<td>Carboxyl group</td>
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<td><em>Litopenaeus vannamei</em> mitochondrial cytochrome C oxidase subunit VIa precursor, mRNA</td>
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<td>cox VI c</td>
<td><em>Litopenaeus vannamei</em> mitochondrial cytochrome C oxidase subunit VIc precursor, mRNA</td>
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<td>CP</td>
<td>Crude protein</td>
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<td>CpG</td>
<td>CpG dinucleotides; cytosine-phosphate-guanine</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNMTs</td>
<td>DNA methyltransferases</td>
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<tr>
<td>dph</td>
<td>Days post hatching</td>
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<td>EAA</td>
<td>Essential amino acid</td>
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<td>ef1α</td>
<td><em>Litopenaeus vannamei</em> elongation factor 1-alpha mRNA</td>
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<td>F, 1-6P</td>
<td>fructose 1, 6-bisphosphate</td>
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<td>fructose 6-phosphate</td>
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<td>free amino acid</td>
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<td><em>Litopenaeus vannamei</em> fructose 1,6-bisphatase mRNA</td>
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<td>Fishmeal</td>
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<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>hk</td>
<td><em>Litopenaeus vannamei</em> hexokinase mRNA</td>
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<td>HUFA</td>
<td>unsaturated fatty acids</td>
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<td>L</td>
<td>Liter</td>
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<td>ldh</td>
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<tr>
<td>luciferase</td>
<td>luciferase control RNA (Promega, L4561)</td>
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<td>Term</td>
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<td>lvglut 1</td>
<td><em>Litopenaeus vannamei</em> glucose transporter 1 mRNA</td>
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<td>lvglut 2</td>
<td><em>Litopenaeus vannamei</em> glucose transporter 2 mRNA</td>
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<td>NEAA</td>
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<td>-NH₂</td>
<td>Amine group</td>
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<td>Mysis stage</td>
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<td>m-2</td>
<td>Square meter</td>
</tr>
<tr>
<td>m-3</td>
<td>Cubic meter</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NFE</td>
<td>Nitrogen Free Extract</td>
</tr>
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<td>O₂</td>
<td>di-oxygen</td>
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<tr>
<td>PC</td>
<td>pyruvate carboxylase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
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<tr>
<td>pepck</td>
<td><em>Litopenaeus vannamei</em> phosphoenolpyruvate carboxykinase gene</td>
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<td>inorganic phosphate</td>
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<td>pk</td>
<td><em>Litopenaeus vannamei</em> pyruvate kinase mRNA</td>
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<tr>
<td>PL</td>
<td>Post-larvae</td>
</tr>
<tr>
<td>PL g⁻¹</td>
<td>Number of post-larvae per gramme</td>
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<tr>
<td>preamylase</td>
<td><em>preamylase 1 (Litopenaeus vannamei)</em></td>
</tr>
<tr>
<td>PUFAs</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SBM</td>
<td>Soybean meal</td>
</tr>
<tr>
<td>SGR</td>
<td>Specific growth rate</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
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<td>TAG</td>
<td>triacylglycerides</td>
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<td>trypsin</td>
<td>trypsin (<em>Litopenaeus vannamei</em>)</td>
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<tr>
<td>TSS</td>
<td>transcription start sites</td>
</tr>
<tr>
<td>Z</td>
<td>Protozoa stage</td>
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</table>
LIST OF FIGURES

Chapter 1
Figure 1.1 Global aquaculture production of *L. vannamei* (adapted from FAO, 2015) 6
Figure 1.2.1-1 External anatomy of penaeid shrimp (adapted from Castex, 2009). 10
Figure 1.2.1-2 Open circulatory system of penaeid shrimp (adapted from Bachère *et al.*, 2004). 10
Figure 1.2.1-3 Life cycle and spatial distribution of penaeid shrimp (adapted from Wickins and Lee, 2002; Whetstone *et al.*, 2002). 14
Figure 1.4.1-1 Drawing of the digestive tract of penaeid shrimps. (adapted from Dall *et al.*, 1990) 30
Figure 1.4.1-2 Drawing of the digestive gland of penaeid shrimps (adapted from Ceccaldi, 1989). 30
Figure 1.4.2.2-1 The general pathways of carbohydrate metabolism, glucose transport and regulation in crustaceans (Adapted from Wang *et al.*, 2016). 34
Figure 1.4.2.2-2 Flow chart of the glycolysis and gluconeogenesis pathways, and the enzymes/substrate involved on each step (adapted from Rocha, 2015). 36
Figure 1.5.2-1 Possible examples of stimuli for nutritional programming in shrimp based on the concept of “metabolic programming” for mammals and fish. 52
Figure 1.5.2-2 The concept of nutritional programming (Lucas, 1998) and stimulus (feed restriction) used on this thesis. 52

Chapter 3
Figure 3.1.1 Embryo and larval stages during early development of *L. vannamei*. Adapted from Wei *et al.*, (2014a). N: Nauplii; Z: Protozoea; M: Mysis; P Post-larvae 62
Figure 3.1.4 Representation of the RT-qPCR. A: Amplification curve; number of cycles necessary to produce the maximum number of DNA double band, which emits a fluorescence signal through the presence of SYBR Green reactive; B: Melting peak, specificity of amplification reaction (oligonucleotides). 68
Figure 3.2 − 1 Experimental rearing tanks used for the hatchery and nutritional programming at shrimp protozoea phase. Laboratory of Aquatic Animal Nutrition LANOA – LABOMAR/UFC (Eusébio, Brazil). 68
Figure 3.2 − 2 Overview of 40 experimental tanks of 1,500 L operated on green-water used for the nutritional programming at post-larval phase. Laboratory of Aquatic Animal Nutrition LANOA – LABOMAR/UFC, Eusébio, Brazil. 70
Figure 3.3.1 Overview of the experimental design from the early nutritional stimulus at the protozoea stage up to juvenile stage. All sampling was carried out as a pool of the whole shrimp body. §: evaluation of growth performance. 72
Figure 3.2.2 Production of the microalgae *Thalassiosira* spp. This microalgae was used as live feed from the late naupli N5/early protozoea Z1 sub-stages to post-larvae PL3. 72
Figure 3.4.1 Schematic overview of the feed restriction stimulus up to the dietary challenge in juvenile shrimp. All the different sampling are presented: whole body were collected at the 1st and 2nd samplings; hepatopancreas and muscle were extracted at the 3rd and 4th samplings. 76
Figure 3.4.3 Experimental diets. 82
Figure 3.4.4 Manual feeding allowance using a PVC feed tray, 200 mm diameter. 84
Figure 3.4.5.1 Illustration of hemolymph withdraw. Source Patricia Vieira. 84
Figure 3.4.5.3 Hepatopancreas sampling. Source: Patricia Vieira 86

Chapter 5
Figure 5.1-1 Targeted window (for the programming) of the protozoa: (Z1-Z2-Z3) of the early development of L. vannamei, characterized by high molecular plasticity (Adapted from the publication 1). gs; glutamine synthase.

Figure 5.1-2 Targeted window (for the programming) of the post-larvae stage 1 PL1 of the early development of L. vannamei (Adapted from the publication 1). pepck: phosphoenolpyruvate carboxykinase; lvglut1: L. vannamei glucose transporter 1.

Figure 5.2-1 Different levels of feeding restriction at the early development of L. vannamei: 70% at the post-larvae stage and 40% at the protozoea stage.

Figure 5.2-2 Summary of the direct effect on the early feed restriction at two sensitive windows (protozoea 40% and post-larvae 70%) on the metabolism.

Figure 5.3-1 Causes and consequences of the early nutritional stimulus on the nutritional programming and development of the non-communicable diets on mammals (adapted from Duque-Guimarães and Ozanne, 2013).

Figure 5.3.1-1 Summary of the data obtained in the thesis about the nutritional programming on L. vannamei linked to the 70% feed restriction stimulus at the early post-larvae stage. FBW: final body weight; SGR: specific growth rate; gs: glutamine synthetase; cox VI b; mitochondrial cytochrome C oxidase subunit VIb; lvglut 1 and 2: glucose transporter 1 and 2; pk: pyruvate kinase (adapted from publication n°2).

Figure 5.3.2-1 Summary of the data obtained in the thesis about the nutritional programming on L. vannamei linked to the 40% feed restriction stimulus at the early protozoea sub-stage Z1 sub-stage. ATP synthase subunit alpha and beta; ldh: lactate dehydrogenase ; pk: pyruvate kinase and hk: hexokinase (adapted from publication n°3).

Figure 5.4-1 Summary of the effect of diet challenge on the growth performance and gene expression of RNA extracted from hepatopancreas. CBH: carbohydrate; CP: crude protein FBW: final body weight; FCR: feed conversion ratio; ldh: lactate dehydrogenase and pepck: phosphoenolpyruvate carboxykinase.

Figure 5.5-1 Summary of the interactions between the nutritional history and the diets on the final body weight FBM (g) of L. vannamei; cox VI c: mitochondrial cytochrome C oxidase subunit VI c; lv glut 1: glucose transporter 1 and Nutritional history (CTL: control; black box and RES feed-restricted group; white box). P43: 43.3% CP and 29.3% CBH; P37: 36.9% CP and 37.6% CBH and P30: 29.5% CP and 45.5% CBH.

Chapter 6

Figure 6.1.1.1 Summary of the possible future research areas for programming shrimp.
LIST OF TABLES

Chapter 1
Table 1.1 Characterization of farming shrimp systems based on production parameters (adapted from FAO, 2006). 8
Table 1.2.1 Developmental stages of penaeidae shrimp larva (adapted from Motoh, 1985; Treece and Fox, 1995; Wickins and Lee, 2002, Juarez and Moss, 2010). Dph, days post-hatching 12

Chapter 3
Table 3.1.2 List of the primers used for real time PCRs. 65
Table 3.3.2 Stimulus Restriction Feeding Protocol for L. vannamei (during 4 days from protozoea Z1 sub-stage up to the Z3 sub-stage). CTL: control group. RES: feed-restricted group (40% of reduction of the feed allowance of the CTL group). 74
Table 3.4.2 Feeding protocol (g of diet per day) used for stimulus of post-larval L. vannamei (between PL1 and PL4 stage). CTL: control group. RES: feed restricted group (70% restriction). 78
Table 3.4.3 Ingredient composition (% as is) and proximate composition (%) DM basis) of experimental diets used during the dietary challenge. 80

Publication 3
Table 1 Stimulus Restriction Feeding Protocol for L. vannamei (during 4 days from protozoea Z1 sub-stage up to the Z3 sub-stage 130
Table 2 Direct effect of the early feed restriction (stimulus) on the mRNA levels (normalized by the reference gene ef1a) of the genes coding digestive, intermediary and energy metabolism measured at the whole body level of L. vannamei protozoea sub-stage 3 (Z3). 131
Table 3 Performance of post-larval L. vannamei in the hatchery culture phase, i.e., from N3 stage up to PL12 stage (hatchery) and from N3 stage up to the PL 29 stage (juvenile; nursery). 132
Table 4 Effect of the early feed restriction (Nutritional History) on the mRNA levels (normalized by the reference gene ef1a) 133
Table 5 The long-term effect of the early stimulus on the mRNA levels (normalized by the reference gene ef1a) of the digestive and intermediate measured on the whole body 134

Chapter 5
Table 5.4 Formula cost of diets used in the challenge phase and the revenue. Values were estimated considering the exchange rate of $ 1.00 USD = R$ 3.08 BRL and Brazilian market shrimp price R$ 29.00 BRL/kg; 9.0 g body weight mean, on 17th February 2018 152
Chapter 1

Introduction
1.1 Aquaculture and shrimp production

1.1.1 History of aquaculture and with emphasis on shrimp farming

In 2014, aquaculture produced 73.8 million MT, with an estimated value of US$160.2 billion. Marine shrimp accounts for 22.6% of the total aquaculture value, but corresponds to less than 10% of the global aquaculture output (FAO, 2016). Together, fisheries and aquaculture products represent an important source of food for humans all over the world. Per capita fish consumption increased from an average of 9.9 kg in the 1960s to more than 19.3 kg in 2014 and 2015. This increment was mainly driven by aquaculture expansion which now responds to more than half of world’s fish consumption. Comparatively, fisheries production has remained relatively stable in the past three decades (FAO, 2016). It is important to note that aquaculture production is highly regionalized. Indeed, Asian countries together are the largest producers. China per se represents 60% of the world aquaculture output, playing a key role in this animal production sector.

Shrimp aquaculture production progressed in similar ways to the terrestrial animal husbandry, i.e., a traditional culture of wild animals at low density in a natural setting which was developed to an intensive culture of domesticated animals under controlled settings. Shrimp farming originated in Asia in earthen ponds intended to the production of milkfish, mullets and other finfish. Incidental wild shrimp fry stocking brought by tide resulted on a small shrimp production 100-200 kg/ha/year (Chamberlain, 2010).

In order to improve the large production of marine shrimp, several species were experimentally studied and some have been selected, i.e., the black tiger shrimp, Penaeus monodon, in Asia (Chamberlain, 2010) while in America, the Pacific whiteleg shrimp, Litopenaeus vannamei, was more successful (Lightner, 2011).

Farming of the whiteleg shrimp began in the Americas on the second half of the 20th century through simple culture practices in earthen ponds. Culture was totally dependable on wild post-larvae capture, naturally abundant on the Pacific (Central and South America) coasts. However, this approach has significant limitations mainly because of the seasonal seed availability of shrimp during El Niño years. A key factor that favored the growth of commercial shrimp production was the knowledge on their life cycle, nutrition and the production of post-larvae from broodstock which spawned in laboratory. This increase seed availability which allowed expansion in production (Boyd, 2006; Jones et al 1997; Le Moullac et al 1992).
1.1.2 Shrimp Farming

Shrimp are farmed globally, but major production countries are in Southern Asia and Central and South America. The technique used for shrimp production is region-related. In the Americas, semi-intensive farms can occupy large areas, at times exceeding 100 ha. Conversely, farms located in the Asian continent are smaller (less than 5 ha) and are normally managed by families and operated under intensive conditions (Boyd, 2006). Production from countries in the Americas accounts for 2.3% of the global output, with Ecuador, Mexico and Brazil acting as the major producers (FAO, 2016; Lightner, 2011).

Important studies about shrimp larvae production were carried out, opening the door to modern farming shrimp production. The first assay was performed with the Kuruma prawn, *Marsupenaeus japonicus*, by the Japanese biologist, Dr. M. Fujinaga in 1933. Moreover, Dr. J.C. Pearson, in the 1930s, described for the first time the eggs of western hemisphere penaeid shrimp as well as the life histories of some American penaeids. Harry Cook and others researchers at the National Marine Fisheries Service laboratory (Galveston, Texas) established the Galveston system for rearing shrimp larvae, which expanded shrimp hatchery technologies (Whetstone *et al.* 2002). This system is characterized by small conical tanks with aeration supply, daily water exchange and addition of exogenous feed and live food (*Artemia* nauplii and microalgae).

The development of the hatchery technology, the widespread on this knowledge and the shift from black tiger to the whiteleg shrimp, turned China into the largest global producer. Nevertheless, the propagation of some viral diseases strongly affected the shrimp farming industry in the 1990s forcing farmers to modify methods in the production. Wild broodstock were identified as vector of diseases, thus commercial hatcheries started to produce their own “domestic” broodstock. In the Americas, the use of wild broodstock dropped down from 80% in 1992 up to 3% in 2001 (Kawahigash, 1992, Moss *et al.*, 2006, FAO 2006, Juarez and Moss, 2010).

These modifications of broodstock access associated with an application of biosecurity programs, certified these domestic broodstock to be free disease shrimp or SPF (Specific Pathogen Free). The term SPF is related within the level of biosecurity and disease history of the facility where shrimp are maintained and raised (Moss *et al.*, 2006, Lightner *et al.*, 2009). Due to diseases outbreaks in *Penaeus monodon* farming in Asia associated with the large scale availability of SPF larvae has led farmers to switch to *L. vannamei*. This species has exceeded the global production of black tiger shrimp in 2003 (FAO 2015).
Figure 1.1. Global aquaculture production of *L. vannamei* (adapted from FAO, 2015).
Thus, all of these aspects allowed an almost 15-fold increase in the global production of farmed shrimp (Figure 1.1). *L. vannamei* shrimp production dropped from 193,000 MT in 1998 to 143,000 tones in 2000 mainly because of outbreaks of WSSV (White Sport Syndrome Virus) in the Americas. Since then, the global production has grown from 270,000 MT in 2004 and to over 3,000,000 MT in 2012.

All of this production is far away from the initial production of 8,000 MT in 1980 (Figure 1.1; FAO 2006; Juarez and Moss; 2010; FAO 2015).

The growth of farm-reared shrimp production has aroused the interest of many people. Farmers looking at increasing their profit have intensified shrimp production. However, many farmers carried out production without any adequate technology unbalancing the environment and creating stressful rearing conditions which have led to the emergence of many different diseases, e.g., viral diseases such as the White Spot Disease (WSD), Syndrome Taura (TSV), Yellow Head Virus (YHV) and Infectious Hypodermal and Hematopoetic Necrosis (IHHNV) or bacterial diseases such as Acute Hepatopancreatic Necrosis Syndrome (AHPNS), commonly known as Early Mortality Syndrome (EMS) and Necrotizing Hepatopanreatitis Bacterium (NHP-B) (Lightner, 2005; Crabtree *et al*., 2006; Lightner *et al*., 2012; FAO 2014). These diseases have caused losses in shrimp production worldwide. Recently, there were events EMS outbreaks in Asia (Thailand, Vietnam Malaysia and China), Mexico and South America. The causative agent of EMS has been reported to be a pathogenic *Vibrio parahaemolyticus* (FAO, 2014; Tran *et al*., 2013). This EMS disease normally affects shrimp post-larvae between 20 and 30 days after pond stocking and frequently causes up to 100% mortality. The first occurrence was reported in southern China in 2010, subsequently in Vietnam, Thailand and Malaysia (FAO 2013). Estimated losses in Asian shrimp production due to EMS reached close to US$ 1 billion (Lightner *et al*., 2012; Tran *et al*., 2013).

Globally, shrimp farming production is characterized by the use of different rearing systems, stocking densities, pond management technology and types of feeds delivered (Table 1.1).

The animal species used in the present thesis is *Litopenaeus vannamei* shrimp. The followings sections will now describe the biology of this shrimp species.
Table 1.1. Characterization of farming shrimp systems based on production parameters (adapted from FAO, 2006).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Extensive</th>
<th>Semi-intensive</th>
<th>Intensive</th>
<th>Super-intensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of pond</td>
<td>Earthen ponds (irregular, tidal areas)</td>
<td>Earthen ponds</td>
<td>Earthen ponds, and/or lined (enhance water quality)</td>
<td>Lined ponds or raceways (greenhouse)</td>
</tr>
<tr>
<td>Pond area (ha)</td>
<td>5-10 up to &gt; 100</td>
<td>1-5 / water depth 1.0-1.2 m</td>
<td>0.1-1 / water depth &gt;1.5 m</td>
<td>&lt;0.2 / Small (282 m²)</td>
</tr>
<tr>
<td>Water exchange</td>
<td>Minimal, tide</td>
<td>Regular water pumping</td>
<td>Applicable Probiotics</td>
<td>Replacement due to evaporation</td>
</tr>
<tr>
<td>stocking densities (PL/m²)</td>
<td>Wild seed, 4-10 (1980)</td>
<td>10-30</td>
<td>60-300 (SPF and SPR¹)</td>
<td>300-450 juveniles (SPF)</td>
</tr>
<tr>
<td>Artificial aeration</td>
<td>Not apply</td>
<td>Minimum</td>
<td>Heavy</td>
<td>Very high</td>
</tr>
<tr>
<td>Fertilization of ponds</td>
<td>Little or not apply</td>
<td>Applicable</td>
<td>1 hp/400-600 kg</td>
<td>DO&gt; 5.0 mg/L</td>
</tr>
<tr>
<td>Formulated feed/Feeding frequency</td>
<td>Low protein 1/day</td>
<td>Supplementation 2-3/day</td>
<td>Applicable</td>
<td>C:N</td>
</tr>
<tr>
<td>Yield (MT/ha/crop)</td>
<td>0.15-0.5</td>
<td>0.5-2.0</td>
<td>Up to &lt; 35</td>
<td>Complete/&gt;8/day</td>
</tr>
<tr>
<td>Cycles (crops)</td>
<td>1-2/year</td>
<td>2/year</td>
<td>2-3/year</td>
<td></td>
</tr>
<tr>
<td>Harvested body weight</td>
<td>11-12 g</td>
<td>4-5 months</td>
<td>16-26g</td>
<td></td>
</tr>
<tr>
<td>FCR</td>
<td>4-5 months</td>
<td>-</td>
<td>&lt;1.2-2.6</td>
<td></td>
</tr>
<tr>
<td>Notes</td>
<td>Latin America</td>
<td></td>
<td>Asia and Latin America</td>
<td>USA 1.5 g/week</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Far from ocean and low salinity</td>
<td>55-91.5% Biosecurity and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>inexpensive areas</td>
<td>eco-friendly</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heterotrophic BFT²</td>
<td></td>
</tr>
</tbody>
</table>

¹SPF – Specific Pathogen Free; SPR – Specific Pathogen Resistant. ²Biofloc system – adjustments of C:N (10:1) ratio to enhance the heterotrophic bacteria population.
Figure 1.2.1. External anatomy of penaeid shrimp (adapted from Castex, 2009).

Figure 1.2.1-2. Open circulatory system of penaeid shrimp. Vascular system (bleu) is continuous with the hemolymph sinuses and irrigates all the body cavity and the sites hematopoiesis. The hematopoietic tissue consists of densely packed of lobules (gray) located at different parts of the shrimp anterior region: hematopoietic dorsal lobules (HDL), hematopoietic ventral lobules (HVL), and the hematopoietic antennal lobules (HAL). H, Heart; HP, hepatopancreas; LO, lymphoid organ and VS, vascular system (adapted from Bachère et al., 2004).
1.2 The biology of *Litopenaeus vannamei*

1.2.1 Life cycle, ontogenesis, feeding habits, morphological characterization and growth pattern of *L. vannamei*

Commonly known as whiteleg shrimp or Pacific white shrimp, *Litopenaeus vannamei* belongs to the *Arthropoda* phylum, which contains the largest number of species. The *Arthropoda* phylum is characterized by the presence of paired appendages (lateral body symmetry) and the existence of a protective carapace called exoskeleton which covers the whole animal. The first report of *Panaeidea* was in 1759 by Seba Amsterdam who named and designed a North American *Panaeidae*. In 1815, Rafinesque recognized them as a distant family between the order *Decapoda*. He suggested a modification to that family and named it as Penedia, which after was corrected as Penaeidae in 1955. Later, Pérez-Farfante and Kensley have established another modification in 1997, changing the taxonomy of the genus of some *Penaeideas* to *Litopenaeus*, *Fenneropenaeus*, *Marsupenaeus* and *Farfantepenaeus*. Thus, the species *Penaeus vannamei* is finally recognized as *Litopenaeus vannamei* (Pérez-Farfante and Kensley, 1997; Treece, 2000; Carvalho, 2011).

The external anatomy of penaeid shrimp can be divided mainly into two sections. The frontal part called cephalothorax is characterized by the presence of a rigid structure called rostrum, and the terminal part the abdomen (Figure 1.2.1-1). On the cephalothorax are located the organs related with the digestive system (hepatopancreas/digestive gland; heart and gills as well as appendices which have several functions (antennal flagellum, antenular flagellum, maxilla and maxillipeds, and pereopods). Moreover, in the abdomen are located the pleopods responsible for swimming (Dall, 1990). In crustaceans, tissue growth is continuous while the “external” is cyclical due to a series of moults (*i.e.*, ecdysis) during which the animal casts off the old exoskeleton (exuvia) (Hartnoll, 1983). This is a fundamental process for growth in crustaceans. The typical weight gain patterns in fishes and crustaceans are described by a sigmoid curve, characterized by a rapid growth in the early stages, decreasing by the aging and sexual maturation (Gulland, 1969). The whiteleg shrimp have the same pattern, moulting process for youngsters are faster than older stages (Cesar, 2006). The cycle of moulting is a repetition of cyclic stages compromising (pre-moult, moult, post-moult and intermoult phases), distinguished by exoskeleton hardness or changes in the organization of the setae from uropods (Chan *et al.*, 1988; Cesar *et al.*, 2006).
Table 1.2.1. Developmental stages of penaeidae shrimp larva (adapted from Treece and Fox, 1995; Wickins and Lee, 2002, Juarez and Moss, 2010). Dph, days post-hatching.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Instar</th>
<th>Description</th>
<th>Time/Days (days post-hatching)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. vannamei</td>
<td></td>
<td>Considering 30-32°C</td>
</tr>
<tr>
<td>Nauplii</td>
<td>E-N</td>
<td>Hatching</td>
<td>20 h (0 dph)</td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>Body pear-shaped</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Beginning of hatchery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Oscellus, mandibles and frontal organs present</td>
<td>20 – 24 h (2 dph)</td>
</tr>
<tr>
<td>Protozoea</td>
<td>N-Z</td>
<td>Metamorphosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z1</td>
<td>Abdomen, Central eyes, first external feeding</td>
<td>1 day (3 dph)</td>
</tr>
<tr>
<td></td>
<td>Z2</td>
<td>Eyes stalked, rostrum, supraorbital forked</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z3</td>
<td>Pair of uropods developed, spines appears on the last abdominal somite</td>
<td>1 day (4 dph)</td>
</tr>
<tr>
<td>Mysis</td>
<td>M1</td>
<td>Shrimp-like body structure, swim head-down</td>
<td>1 day (6 dph)</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>vigorous movements of the tail</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M3</td>
<td>Pleopods bubs present but unsegmented, curved</td>
<td>1 day (7 dph)</td>
</tr>
<tr>
<td></td>
<td>M-PL</td>
<td>Pleopods elongated and segmented</td>
<td>1 day (8 dph)</td>
</tr>
<tr>
<td>Post-larvae</td>
<td>M-PL</td>
<td>Metamorphosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PL1</td>
<td>Claws dactyls/pereiopods, pleopods fully developed</td>
<td>1 day (9 dph)</td>
</tr>
<tr>
<td></td>
<td>PL2</td>
<td>1 spines mean 2-3 days, developmental of gills</td>
<td>1 day (10 dph)</td>
</tr>
<tr>
<td></td>
<td>PL3</td>
<td>Presence of spines at the rostrum</td>
<td>1 day (11 dph)</td>
</tr>
<tr>
<td></td>
<td>PL4</td>
<td>Benthonic behavior, good development of gills – possibilities to adapt to different salinities</td>
<td>1 day (12 dph)</td>
</tr>
<tr>
<td></td>
<td>PL5</td>
<td>Commercialization</td>
<td>1 day (13 dph)</td>
</tr>
<tr>
<td></td>
<td>PL6</td>
<td></td>
<td>1 day (14 dph)</td>
</tr>
<tr>
<td></td>
<td>PL7</td>
<td></td>
<td>1 day (15 dph)</td>
</tr>
<tr>
<td></td>
<td>PL8</td>
<td></td>
<td>1 day (16 dph)</td>
</tr>
<tr>
<td></td>
<td>PL9</td>
<td></td>
<td>1 day (17 dph)</td>
</tr>
<tr>
<td></td>
<td>PL10</td>
<td></td>
<td>1 day (18 dph)</td>
</tr>
</tbody>
</table>
This process is regulated by both stimulating and inhibiting hormones located in the Y-organ (in thorax) and X-organ (in the eyestalk), respectively (Chang, 1995).

Several physiological modifications are associated with the moulting cycle (Chang, 1995; Promwikorn et al., 2004), affecting the nutritional status during moulting cycle. It is necessary to build up nutrient reserves before and start feeding again in the post-moult stage as crustaceans fast during moulting. Thus, animals on intermoult are desirable for use on nutritional studies (Cousin, 1995). The penaeids, like most of the arthropods, have an open-circulatory system where the hemolymph, correspondent to the blood of vertebrates (Figure 1.2.1-2), circulates. Shrimp have a muscle heart located dorsally in the cephalothorax. The hemolymph has a blue coloration because of hemocyanin, a copper-based protein which correspond to up to 90% of the total protein present on the plasma. The hemocyanin has an important role as oxygen carrier, nutrient transport and participation on the immune defense (Barracco et al., 2014).

*L. vannamei* is native from eastern Pacific coast of Central and South America from Sonora in Mexico to Tumbes in northern Peru. It has been introduced all over the world since the 1970s, especially by its introduction as the farmed shrimp in Asia. It became the principal shrimp species in aquaculture in 2000 and corresponds to the 2/3 or more of the total the global shrimp production since 2014. This shrimp species is predominantly benthic, characterized as omnivorous or detritivores. The whiteleg is classified as euryhaline shrimp species, which tolerates a large range of salinity (0.5 to 50.0 g/L). However, the best growth rate for this species is found at intermediated salinities (FAO, 2015), between 20 to 25 g/L. Literature data have shown that modifications on the ambient salinity affect the growth performance and physiological responses of this species (Li et al., 2007, 2008).

During the early development (different steps called embryo, nauplii, protozoa, mysis and, post-larvae), the whiteleg shrimp, as all the crustaceans, goes through a typical metamorphosis process within different stages before becoming a juvenile and an adult shrimp (Table 1.2.1).

Modifications of the physiology, morphology, feeding and swimming habits are associated with the different developmental stages (Figure 1.2.1-3). The first stage after spawning is the nauplii stage, characterized by five sub-stages and reabsorption of yolk reserves. When they reach protozoa stage, their feeding habits change to herbivorous (first feeding), filtering the unicellular microalgae from the water. There are three protozoa substages during 3-4 days until the next metamorphoses up to mysis stage.
Figure 1.2.1-3. Life cycle and spatial distribution of penaeid shrimp (adapted from Wickins and Lee, 2002; Whetstone et al., 2002).
The mysis stage is characterized by its feeding habit, acting as a real carnivorous, feeding voraciously on zooplankton (rotifers, Artemia nauplii, copepods). This stage lasts from 3-5 days and has three substages. When shrimp reaches the next metamorphose, they become post-larvae (PL) or juvenile which are similar to the adult form. By that point, they start to develop feeding habits similar to adults, i.e., omnivorous, detritivorous and/or scavengers. Other important aspect for shrimp is the development of the gill structures, crucial for a better osmotic regulation and more efficient gas exchange, which are improved step by step (Juarez and Moss, 2010). Gills morphological characteristics are useful for commercial hatcheries. When PL reaches the full gill development stage, they begin to be acclimated to different water salinities. Similar to adult shrimp, shrimp adopts benthic habits (lives on the substrate), around the 5-6th day after the metamorphosis from mysis to PL (Wickins and Lee, 2002; FAO, 2015).

1.2.2 Life cycle and feeding differentiation

During early development, as nauplii stage, shrimp have their own yolk sac with nutrient reserves. They do not need to swim for food search, so they channel all their energy reserves to their development and growth (moulting). Nauplii swim intermittently and are phototactic, used in commercial hatcheries during harvest. The name “nauplii” is characteristic to crustacean larvae which use their frontal appendages, antennules and antennae, originating from the head (FAO, 2015, Juarez and Moss, 2010).

The following larval stages, protozoea and mysis, shrimp ingest phytoplankton and zooplankton, respectively. Protozoea shrimp have a better swimming behavior, but remain planktonic. Under natural conditions, wild larvae are carried towards shallows areas (estuary) by the currents. Protozoea can swim continuously, pursuit their food and have a high level of food ingestion, which can reach up to 8 fold of their own weight. When the mysis stages is reached, they swim head-down with vigorous movements of the tail. Strong mysis tend to swim towards the water surface. Post-larval (PL) shrimp exhibit a different swimming behavior. They are able to go forward (chase the prey) and also able to maintain their position in the water column, mainly because of the full development of their pleopods or swimming appendages (Juarez and Moss, 2010). From juvenile to adult stage, it can take from 180 to 300 days (Figure 1.2.1-3). Shrimp can be called adults when they reach sexual maturity (Wickins and Lee, 2002).
1.3 Shrimp Nutrition

1.3.1 Diets for farmed shrimp and nutritional requirement

In shrimp farming production relies on the use of industrially manufactured feeds, which represent up to 60% of the total production costs (Hertrampf and Piedad-Pascual, 2000; Sookying et al., 2013). Due to the high impact on production costs, aquaculture nutritionists develop highly digestible feeds to meet shrimp nutrient requirements to result in the best growth performance, the lowest impact on the environment and a highest flesh quality. Contrary to terrestrial husbandry, aquaculture nutritionists must deal with a wide range of challenges associated with the aquatic environment, i.e., how to monitor feed intake, feed delivery and leaching of feed nutrients. Grower feeds for marine shrimp are typically high in crude protein (CP) with levels exceeding 35% (NRC, 2011; Nunes et al., 2014). Historically, these diets used large amounts of expensive and constrained fish meal as a source of digestible protein. However, the aquaculture industry has been pressures to reduce its dependence on marine-based ingredients, mainly fish meal and fish oil (Klinger and Naylor, 2012). Over the past decade, due to its high and volatile prices and lower market availability of the fish meal, byproducts obtained from agriculture, fisheries and the slaughtering of terrestrial production animals have become the protein sources of choice in the industry (Gatlin et al., 2007; Tacon and Metian, 2008; Nunes et al., 2014; FAO, 2016).

Under natural conditions, penaeid shrimp are dependent on food particles rich on proteins (i.e., crustaceans, small invertebrates and mollusks). Aquafeeds are produced with several raw materials, similar to those used in the husbandry of land animals. Nevertheless, as explained before, aquatic species require diets with a high dietary protein levels (Dall et al., 1990; Nunes et al., 2014). This high dietary protein requirement is mainly related to the fact that protein and AA (amino acids) can be catabolized as energy sources (Rosas et al., 1995, 2002). Indeed, as reported to whiteleg shrimp reared in low salinity, the high dietary protein content significantly improves their growth performance (Cuzon et al., 2004; Li et al., 2011; Wang et al., 2015, 2016). Nevertheless, proteins (amino acids) should not be used as energy due to their cost and is not environmental friendly (Singh et al., 2006; Shahkar et al., 2013). In animal nutrition research, the increase of non-protein ingredients as energy sources (dietary lipids and carbohydrates, CBH) has been shown to have a protein-sparing effect (Cho and Kaushik 1990). However, this protein sparing effect is mainly dependable of the animal species and their feeding habit (NRC, 2011; Wang et al., 2015).
1.3.1.1 Protein and amino acid requirement

The protein dietary requirement plays an important role on penaeid shrimp since they are a limiting nutrient for growth (synthesis of new muscle tissue). Although, aquatic species are better convertors of dietary proteins compared with terrestrial animals, dietary protein content, availability and digestibility must be considered, both for sustainability as well as for environmental issues. Excess of nitrogen excretion can affect water quality and lead to eutrophication (Cho et al., 1994; Kureshy and Davis, 2002; Kaushik and Seiliez, 2010; Klinger and Naylor, 2012; Nunes et al., 2014). Historically, fishmeal has been used as the best protein source even though several other protein ingredients can be used in aquafeeds. Fish meal is used due to its high nutrient value, digestibility and essential amino acid (EAA) profile, which perfectly meets shrimp requirements (Nunes et al., 2014).

The amino acids that an organism cannot synthesize on its own are referred to be EAA. The shrimp *L. vannamei* requires the same 10 EAAs as the other species, *i.e.*, arginine, methionine, valine, threonine, isoleucine, leucine, lysine, histidine, phenylalanine and tryptophan (Kanazawa and Teshima, 1981). Among the 10 listed EAA, three (methionine, lysine and arginine) are considered as the most limiting in commercial feed formulas, being indispensable for a normal growth and survival of shrimp. In contrast, the nonessential amino acids (NEAA) can be synthesized from precursors by an addition of amino group to a tricarboxylic acid (Coloso and Cruz, 1980, Cowey and Walton, 1989; Akiyama et al., 1991).

On a general way, as with others animals, shrimp does not have a protein requirement per se, they have a need on specific dietary amino acids (AA). Amino acids (AA) supplied by the digestion of dietary protein (diet intake, exogenous source) or catabolism of body protein (endogenous source) enter into the free amino acid (FAA) pool (Cowey and Walton, 1989; Kaushik and Seiliez, 2010). Once digested, AA are released into the hemolymph and delivered to the target organs for protein synthesis. However this mechanism is not clearly understood for shrimp. The FAA is a dynamic status as reflex of the dietary supply, endogenous protein degradation, uptake for synthesis and protein accretion in shrimp (Mente et al., 2002).

AA have several roles in metabolism besides acting as protein building blocks. They play an important role on other biological molecules, such as forming parts of coenzymes, precursors for the biosynthesis of structural molecules and metabolic intermediates (*i.e.*, acetate and pyruvate), which are used as substrate for energy through the neoglicogenese pathway (NRC, 2011). Besides the function of methionine (Met) as an EAA in protein synthesis, Met is a methyl donor group through the conversion on S-adenosylmethionine. SAM which is the major
methyllating agent involved on the DNA methylation, an important mechanism for the epigenetic (Best et al., 2018; see 1.5.3 Mechanisms of global epigenetic and aquaculture). It serves as precursor of several sulphur-containing compounds, such as the synthesis of cysteine (Finkelstein et al., 1988) and glutathione peroxidase, the most important anti-oxidative system in the body (Stipanuk, 2004).

Optimal protein cannot only meet the demand for growth, but also contain AA that improve stress resistance and survival by serving as osmolyte to cope with salinity challenge (Li et al., 2017). Free AA, accumulated in the intracellular compartment, improve osmoregulation for marine invertebrates and the most common FAA are glycine, alanine, proline and taurine (Yancey et al., 1982; Fang et al., 1992).

Dietary protein requirements for penaeidae species range from 30% up to 60% (Akiyama et al., 1992). This high variation is related with their feeding habits, developmental stages and the rearing system applied (Deshimaru and Yone, 1978; Smith et al., 1985; Cousin et al., 1993; Samocha et al., 1993; Velasco et al., 2000). During its juvenile phase, L. vannamei requires a dietary protein range between 30 and 36% (Colvin and Brand, 1977; Smith et al., 1985; Kureshy and Davis, 2002). Protein sources are the most expensive ingredients in shrimp feed formulas. However, excessive dietary protein have many disadvantages and should be considered. The nitrogen (protein) which is wasted in the aquatic environment not only increases production costs, but also leads to increased pollution which causes toxicity from secreted compounds and compromises water quality (Singh et al., 2006; Güroy et al., 2012, Shahkar et al., 2013). Both, unquestionably, will reduce shrimp growth and survival performance. As a result, shrimp nutritionists attempt to avoid excess dietary protein by maximizing use of protein for growth and reducing their contribution in formulation costs (Singh et al., 2006; Nunes et al., 2014). This thesis also aims at improving the use of diets with low protein content through nutritional programming.

1.3.1.2 Lipid requirement

Lipids are considered to be macronutrients as proteins and carbohydrates, and among them, lipids have the highest energy content. However, defining a dietary lipid requirement is more complex since: i) lipid requirement can be influenced by dietary proteins and carbohydrates (CBH), which can serve as energy sources; ii) lipid source can be endogenous through the lipogenesis pathway with AA and CBH as the main source of carbons (NRC, 2011); iii) lipids are a complex family of nutrients such as fatty acids, phospholipids and glycerol. The bulk of
dietary lipids are fatty acids, which serve as an energy source, but also play a key role in metabolism regulation through specific long-chain polyunsaturated fatty acids (LC-PUFA). Comparatively, phospholipids and glycerol are essential components of cell membranes (He et al., 1992; Li et al., 2017; NRC, 2011; Tseng and Hwang, 2008). The most important lipid in animals, including aquatic species, is cholesterol. This nutrient is a member of sterol family tetracyclic hydrocarbon alcohols. Cholesterol is an essential component of all cellular membranes and a precursor of steroid hormones (NRC, 2011; Tocher and Glencross, 2015). The ability to synthesize highly unsaturated fatty acids (HUFA) varies among aquatic animals (Sargent et al., 2002). However, crustaceans have a limited ability to synthesize de novo PUFA (polyunsaturated fatty acids), such as linoleic (18:2n-6, LOA) and linolenic (18:3n-3, LNA) acids, or LC-PUFA (long chain polyunsaturated fatty acids), such as eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids; suggesting these fatty acids are essential (Kanazawa et al., 1979a,b; Kayama et al., 1980; Suprayudi et al., 2004).

The optimal dietary lipid levels for whiteleg shrimp has been evaluated at several water salinities, showing that the optimal dietary lipid content is related to this parameter. Overall, shrimp weight gain responses to different levels of dietary oils indicate that the highest gain is achieved at dietary inclusion levels between 5-6% (NRC, 2011; Li et al., 2017). However, dietary inclusion lipid levels in excess of 10% is related with reduced growth performance due to either a reduction in feed intake or lack of ability to metabolize high dietary lipids levels efficiently. As a consequence, reduced growth has been associated with accumulation of lipids in tissues (González-Félix et al., 2002a,b; NRC, 2011). Nevertheless, under challenging conditions (low water salinity), high dietary inclusions (10-12%) has improved shrimp growth performance (8%), acting as an immune stimulant (Zhang et al., 2013; Li et al., 2017).

### 1.3.1.3 Carbohydrate requirement

Carbohydrates (CBH), compared with the other energy nutrients, are the cheapest energy source in animal feeds. Although it considered as a primary energy source in many animal species, dietary CBH are not efficiently used by aquatic carnivorous species (Wilson and Poe 1987, Wang et al., 2016). Additionally, a high intake of dietary CBH is commonly associated with deleterious effects on growth performance, immunity and survival (Shiau et al., 1991b; Guo et al., 2006). In general, fish and shrimp do not require dietary CBH, and regardless of the species, they are able to survive and growth when fed diets without CBH (NRC, 2011).
This is possible because glucose is highly synthesized from non-glucose precursors (amino acids, lactate), through the gluconeogenic pathway (Cowey and Walton, 1989; Enes et al., 2009).

Although shrimp have limited ability to utilize CBH and cannot adapt to a high level of dietary CBH (Shiau et al., 1991b, Shiau and Peng, 1992), CBH are often included in artificial diets for crustaceans as an energy source to spare protein (Cuzon et al., 2004; Cruz-Suarez et al., 1994). The digestibility of CBH depends on its source, chemical composition, manufacturing (heat process), dietary inclusion and the biological animal status driven by environmental salinity stress (Cuzon et al., 2000; NRC, 2011; Scholnick et al., 2006; Spannhof and Plantikow, 1983; Wang et al., 2014, 2015). The use of diets containing complex CBH (starch) is generally better than then use of high quantities of simple sugars such as glucose, which is rapidly absorbed and released into the hemolymph, leading to prolonged hyperglycemia. While complex CBH require enzymatic hydrolysis for digestion (Alava and Pascual, 1987; Shiau and Peng, 1992; Shiau et al., 1991a, b; Rosas et al., 2000; Rosas et al., 2001b). Literature data reports that a high level of dietary CBH leads to poor growth performance, depressed survival and immunity. However, when digestible CBH is used at dietary inclusions between 20 to 30%, it can spare dietary energy from proteins and lipids (Cuzon et al., 2004; Guo et al., 2006; Gatlin et al., 2007; NRC, 2011; Pascual et al., 2004; Shiau, 1998; Wang et al., 2016). Thus, the development of shrimp feeds containing a lower protein content (especially derived from fish meal) and higher levels of CBH can be beneficial in shrimp nutrition.

1.3.2 Development of novel diets for shrimp aquaculture

The development of modern aquaculture was achieved by improvements on farm management, genetic traits for growth and resistance, disease control and a better understanding on the nutritional requirements of farmed animals (Nunes et al., 2014).

In recent years, shrimp farming production is undergoing an intensification process making it more dependable on complete aquafeeds. Today, the bulk of the marine shrimp production is derived from fed-based systems (Tacon and Metian, 2008).

Ingredients used in aquafeeds are similar to those in land animal feeds. However, the fish meal (FM) is the preferred protein source due its high nutrient value (i.e., high protein digestibility, and EAA profile) and bioavailability. Historically, industrially-compounded shrimp feeds contain levels of FM which can reach up to 15% of the feed formula. Feed costs has a significant contribution to total shrimp production costs (Amaya et al., 2007; Tacon and Metian, 2008;
Along with the environmental pressure to reduce the use of marine proteins in commercial formulations, the stagnation of capture fisheries, and the high and volatile prices has pushed shrimp nutritionists to develop new strategies to reduce the dependency on FM.

The high global production and availability associated with the wide range of products make vegetable ingredients (soybean meal, canola meal and corn gluten meal) strong and interesting candidates for the aquafeed production and FM substitute (Hardy, 2010). Nevertheless, plant ingredients have several disadvantages, such as, the relative low protein content, the low attractability and palatability, the unbalanced EAA profile (methionine, lysine). They may also contain high levels of anti-nutritional factors and a high amount of carbohydrates (Francis et al., 2001; Gatlin et al., 2007, Nunes et al., 2011, 2014). Among the terrestrial plant-based protein sources, soybean meal (SBM) is the most abundant and has received considerable attention as a replacement for FM in aquatic animal feeds because of its availability, relatively well balanced AA profile and consistent nutrient composition. However, the presence of anti-nutritional factors (ANF) such as lectins, oligosaccharides, saponins, and trypsin inhibitors, can limit the dietary inclusion levels of SBM in aquafeeds. Conversely, the use of a mixture of plant-based ingredients can improve the AA profile (Akiyama et al., 1991; Amaya et al., 2007; Chou et al., 2004; Hardy, 1999; Molina-Poveda et al., 2013; Samocha et al., 2004; Sookying et al., 2011; Tacon, 2000).

The following alternative proteins can reduce the reliance of aquafeeds on FM: i) marine proteins, such as seaweeds and microalgae, are potential sources of AA and PUFAs (18:2n-6; 18:n3-3), minerals and vitamins and act as a natural feeding effectors in low FM diets (Qiu, 2017; Radhakrishnan et al., 2014; Rodríguez-González et al., 2014; Silva-Neto et al., 2012; Takeuchi et al., 2002); ii) insect meal (Panini et al., 2018); iii) fisheries by-products and proteins obtained from the slaughtering of terrestrial production animals (Nunes et al., 2014).

Farm management, genetics, disease control and nutrition play an important role in the success of aquaculture production (Nunes et al., 2014). In nutrition (defined as the adequate allowance of nutrients required for growth and metabolism maintenance) all nutrients have their particular importance. However, protein and amino acids have a crucial role in growth, acting as a building block for muscle synthesis, tissue repair and maintenance of normal metabolism (Kureshy and Davis, 2002).
Recent improvements in aquaculture nutrition have enhanced the use of plant proteins, but have not avoided the higher intake of dietary CBH (Klinger and Naylor, 2012; Nunes et al., 2014, 2016; Wang et al., 2016; Li, 2017).

Albeit, *L. vannamei* has a limited ability to use CBH, dietary inclusions of complex starch (lower than 30%) has a positive impact on the protein-sparing effect (Cuzon et al., 2004; Guo et al., 2006; NRC, 2011). Thus, improving the ability of the whiteleg shrimp on the utilization of the dietary CBH, naturally available in plant ingredients, may have positive consequence in the reduction of dietary protein.

1.4 Use of nutrients: digestion process and intermediary metabolism

1.4.1 Digestion and juvenile digestion anatomy

The digestive anatomy of shrimp is divided into three major parts: the foregut (mouth, esophagus and proventriculus), midgut (hepatopancreas and intestine) and the hindgut (rectum and anus) (Figure 1.4.1-1). Food particles are carried into the mouth by external appendices, like the maxilla and maxillipeds. Then, through regular peristaltic contraction movements, they are swallowed and transported into the oesophagus, leading them to the anterior chamber of the proventriculus (stomach).

In the stomach and gastric mill (cardiac and pyloric stomachs, respectively), food particles are ground by the teeth and ossicles (calcified structures) and filtered by the filter bistles (Figure 1.4.1-2). The very fine (few microns) particles continue the digestive process in the midgut. Masticated and filtered food particles are exposed to digestive enzymes secreted by the digestive gland, named as hepatopancreas, which accounts for 2 to 6% of the shrimp body mass. Larger particles are conveyed by fluid streaming retrograde to the more proximal parts of the stomach for further degradation (Ceccaldi, 1989; Richard, 2011; NRC, 2011). Nutrient digestion and absorption takes place in the midgut, mainly with the action of the hepatopancreas. This organ is the principal secretory organ of shrimp, acting as a pancreas, intestine and liver. It is responsible for the synthesis and secretion of digestive enzymes, absorption of digested material, and (intermediary) metabolism of macronutrients and minerals. In addition, the hepatopancreas is the center for the production of materials required for temporally distinct events of ecdysis and vitellogenesis. In the hepatopancreas, two kinds of cells are responsible for synthesis of digestsive enzymes. Digestive enzymes are synthesized by the fibrillar cells (F-cells) and accumulated in the blister-like cells (B-cells) (Vogt et al., 1989).
**Figure 1.4.1-1.** Drawing of the digestive tract of penaeid shrimp. (Adapted from Dall et al., 1990). A, anus; AC anterior chamber of proventriculus – cardiac pocket (stomach); AD, anterior diverticulum of mid-gut; D, digestive gland; M, mouth; MG, mid-gut; O, oesophagus R, rectum.

**Figure 1.4.1-2.** Drawing of the digestive gland of penaeid shrimp (Adapted from Ceccaldi, 1989).
The hepatopancreas secretes a large range of digestive enzymes, like the proteases; lipolytic enzymes; chitinases; cellulases; laminarinase; β/α-glucosidase and/or α-amylase (Carrillo-Farnes et al., 2007; Figueredo and Anderson, 2009; Johnston and Freeman, 2005; Xue et al., 1999). Shrimp fed with high dietary protein levels show high proteinase activity, whereas herbivorous animals (e.g., Macrobrachium spp.) tend to produce a high amount of carbohydrases. Omnivorous opportunistic feeders possess a high activity of several enzymes, so they are able to utilize a wide range of food sources (NRC, 2011). Several carbohydrase enzymes have been detected in the digestive tract and hepatopancreas of shrimp, suggesting they are able to hydrolyze a great variety of oligo and polysaccharides (Carrillo-Farnés et al., 2007; NRC, 2011).

Three alpha-amylase have been cloned from L. vannamei (Van Wormhoudt and Sellos, 2003), suggesting this species could digest carbohydrates.

In general, in most crustaceans, trypsin and chymotrypsin act as the main endopeptidases. However, information about the exopeptidases, like carboxypeptidase and aminopeptidase, is very scarce (Carrillo-Farnés et al., 2007; NRC, 2011). Lipase (glycerol ester hydrolase) is a versatile group of enzymes which hydrolyzes triglycerides esters, but also express other enzymatic activities, such as phospholipase. Several studies have reported that enzyme activity depends on several factors, including feeding strategy, dietary composition, growth stages and moult cycle (Fang and Lee, 1992; Fernández et al., 1997; Jones et al., 1997; Le Moullac et al., 1994; Le Vay et al., 2001). The ontogenetic changes in digestive enzymes have been often used to adjust diets to crustaceans during their life cycle (Lee et al., 1984; Fang and Lee, 1992; Le Moullac et al., 1994).

1.4.2 Intermediary metabolism

Nutrients are required by animals to sustain life processes and metabolism, acting as precursors for biosynthesis of structural or storage molecules, enzymes, metabolic intermediates and other molecules. Part of the nutrients consumed is catabolized to provide chemical energy which is required for use in anabolic and other life-sustaining processes.

Animals metabolize specific nutrients, each with specific roles and metabolic intermediates derived from nutrients used simultaneously in the same process, with several interactions between nutrients (Blaxter, 1989; NRC, 2011). The following sub-chapters describe the main metabolic pathways and the key enzymes/transporters involved in the regulation of these
pathways. In the present thesis, these genes (in bold) were used as molecular biomarkers of the metabolism. It is very important to note here that all these biomarkers are regulated by nutrition and are thus very useful for the investigation conducted.

1.4.2.1 Amino acid metabolism

Amino acids (AA) are organic molecules containing both amine (-NH$_2$) and a carboxyl (-COOH) function groups, with the general formula H$_2$NCHRCOOH, where R is a side chain. AA are classified according to their structure, metabolic fate (gluconeogenic or ketogenic) or linked to their capacity to be synthesized by the organism (essential; non-essential or semi-essential).

The digestion of proteins results in free amino acid (FAA) pools, which are absorbed by hepatopancreas and released into the hemolymph for delivery for protein synthesis. The FAA is in a dynamic status reflecting the dietary supply, endogenous protein degradation, uptake for synthesis and protein accretion in shrimp (Mente et al., 2002; NRC, 2011). The breakdown of amino acids generally occurs in two steps. First, there is the deamination which comprises the removal of the ammonia group. The latter can be either converted to ammonia and secreted (BUN, nitrogen excretions through gill and urine) or transferred to become a glucogenic amino group (Richards, 2011). The second stage is the conversion of the carbon skeletons, the α-keto acids produced by deamination, to citric acid cycles intermediates (Cowey and Walton, 1989). The carbon skeleton has free energy that can be either harnessed by tricarboxylic acid cycle (TCA) or converted into fatty acids (through lipogenesis) or glycogen for the future use as an energy source (NRC, 2011).

Penaeid shrimp are ammoniotelic animals, like teleost, with more than 70% of excreted nitrogen being in the form of ammonia (Regnault, 1987). They exhibit an extremely efficient transfer mechanism for ammonia across their branchial epithelium by passive diffusion and active ionic exchange (Regnault, 1987; Greenaway, 1991).

Ammonia originates from the catabolism of nitrogenous compounds, such as AA (dietary and endogenous AA) and nucleic acids (Regnault, 1987; Greenaway, 1991; Kaushik, 1998). Like in other species, shrimp can derive energy from the degradation of AA which are used either to produce pyruvate and acetyl CoA or enters directly the Krebs cycle (Claybrook, 1983). AA catabolism occurs through two main pathways known as direct deamination and transdeamination, among which only few AA (serine and proline) can undergo the former (Regnault, 1987). Transdeamination, commonly observed in crustaceans (Regnault, 1987), is
Figure 1.4.2.2-1. The general pathways of carbohydrate metabolism, glucose transport and regulation in crustaceans (adapted from Wang et al., 2016).
catalysed by the aminotransferases (alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT)) and the **glutamate dehydrogenase** enzyme (GDH). Aminotransferases have been detected in several penaeid species (Claybrook, 1983, Chien et al., 2003, Pan et al., 2003) with GDH being found in crustaceans (Claybrook, 1983) at various concentrations depending on the species (Batrel and Regnault, 1985; King et al., 1985) and tissues (Chaplin et al., 1970; Li et al., 2009). Aminotransferases catalyze the transfer of the amino group to a ketoacid (resulting principally into L-glutamate formation) (Regnault, 1987), while GDH catalyses the reversible conversion of glutamate into ketoacid and ammonia (Greenaway, 1991). Although, it was first believed that only the reductive function of GDH (ammonia elimination) occurred in shrimp (Regnault, 1987), later studies also demonstrated the oxidative function of GDH leading to ammoniogenesis (Batrel and Regnault, 1985; King et al., 1985; Regnault and Batrel, 1987; Greenaway, 1991). Prior to excretion, some enzymes are thought to play a role in ammonia detoxification (Greenaway, 1991). In crabs, high activity of both **glutamine synthetase** (GS) and glutaminase have been detected (in muscle and gills, respectively), suggesting detoxification through glutamine formation, similarly to mammals (King et al., 1985). However, GDH is considered the key regulator of nitrogen catabolism (Bidigare and King, 1981, King et al., 1985) as its activity accounts for most of the ammonia excretion (Batrel and Regnault, 1985).

Glutamine synthetase (GS) plays an important role on the nitrogen metabolism and catalyzes the ATP-dependent conversion of glutamate and ammonium to glutamine, being critical in the detoxification process of the highly mobile and toxic ammonia (Essex-Fraser et al., 2005). In addition, GS is involved on the recirculation of the neurotransmitter glutamate, synthesis of glutamine for the production of amino acids, sugars and glucosamine-6-phosphate and participation in gametogenesis (Essex-Fraser et al., 2005; Carla et al., 2008). Studies indicate that GS plays an important role in stress resistance and adaptation (Ip et al., 2009; Lai et al., 2011; Tanguy et al., 2005). Essex-Fraser et al. (2005), reported that a high glutamine synthetase gene expression followed by the formation of the active protein are crucial in the early stages of rainbow trout (*Oncorhynchus mykiss*) to convert ammonia into glutamine, which may can be further utilized on other pathways.

### 1.4.2.2 Glucose metabolism

The carbohydrate metabolism pathway in crustaceans are summarized in Figure 1.4.2.2-1. Polysaccharides are hydrolyzed into oligosaccharides, maltose and others smaller sugars in the...
Figure 1.4.2.2-2. Flow chart of the glycolysis and gluconeogenesis pathways, and the enzymes/substrate involved on each step (adapted from Rocha, 2015).
digestive gland of crustaceans (Van Wormhoudt and Favrel, 1988). Finally, these compounds are hydrolyzed in monosaccharides, transported as glucose, and absorbed by other organs. The chemical reactions involved in the Krebs cycle, or the tricarboxylic acid (TCA) cycle, mainly occur in the hepatopancreas and muscle in crustaceans (Boulton and Huggins, 1970). The enzyme activity involved in the TCA pathway is closely related to the glucose content and changes in relation to glucose concentration. In order to balance their cells with the external environment, animals will effectively regulate their blood glucose concentration by hormones, storing excess glucose.

Differently from fish and other vertebrates, crustaceans do not regulate blood glucose through insulin functions. The hormones involved in glucose regulation in crustaceans are crustacean hyperglycemic hormone (CHH), insulin-like peptides and insulin-like growth factor (IGF). Thus, the variation of the hemolymph glycemia is smaller than in fish, due to the cooperative action of these hormones (Furuichi and Yone, 1981; Verri et al., 2001). The CHH plays several roles, such as molting regulation, reproduction and osmoregulation (DeKleijn and VanHerp 1995, 1998; Charmantierdaures et al., 1994; Serrano et al., 2003).

Furthermore, the main function of CHH is its role in the metabolism of FA and CBH, functioning as a regulator of the hemolymph glucose, supplying energy to different organs (Fanjul-Moles, 2006). When the hemolymph glucose concentration is low, CHH mobilizes glucose from glycogen by promoting glycogenolysis and inhibits the glycogen synthesis. On the other side, when there is high hemolymph concentration of glucose, the secretion of CHH is inhibited then insulin-like peptides and IGF are activated (Lee et al., 2014). The IGF polypeptides play a role in CBH metabolism by regulation of hemolymph glucose concentration as a positive feedback to the synthesis of glycogen. The IGFs cannot freely circulate on the circulatory system, and require specific affinity IGF-binding proteins to move into the circulatory system (Rosen et al., 2013; Wang et al., 2016).

The main pathway for glucose metabolism is the glycolysis, which represents the phosphorylation of glucose to pyruvate, and the opposite pathway gluconeogenesis which represents the production of glucose synthesized de novo from non-carbohydrates substrates (AA and FA; Figure 1.4.2.2-2).

Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK), are key regulatory enzymes of glycolysis (Fraenkel et al., 1996). Hexokinase plays an important role on glucose metabolism. HK catalyzes the first step in the oxidative metabolism of hexoses via glycolysis, which consists on the phosphorylation of glucose to glucose-6-phosphate. This molecule is used in other metabolic pathways, such as glycogenesis and the pentose-phosphate pathway...
(Tsai and Wilson, 1997; Enes et al., 2009). The HKs are a family of evolutionary and structurally related enzymes present in eukaryotic cells from yeasts to mammals, which differ in their catalytic and regulatory properties, molecular mass and tissue distribution (Iynedjian, 1993; Cardenas et al., 1998; Wilson, 2003). In mammal tissues, four distinct HK isozymes are reported and named as type I-IV (also called as type 1-4 or A-D) (Cardenas et al., 1998; Wilson, 2003; Enes et al., 2009). Information about hexokinase family in marine crustaceans is very scarce. HK activity in the hepatopancreas has been shown in shrimp (Rosas et al., 2001; Gaxioal et al., 2005) and the HK of L. vannamei cDNA sequence characterized (Soñanez-Organis et al., 2011).

PFK, considered by many authors as the main highly regulated enzyme of the pathway, catalyzes the essentially irreversible Mg ATP-dependent phosphorylation of fructose 6-phosphate (F6P) to yield fructose 1, 6-bisphosphate (F, 1-6P) (Hasawi et al., 2014). PFK is a complex enzyme involved in a rate-determining step of glycolysis and thus represents an essential metabolic control point or node for carbohydrate utilization (Jenkins et al., 2011). PK is a key enzyme of the glycolysis pathway that catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate with the production of adenosine triphosphate (ATP) from adenosine diphosphate (ADP), the last step of glycolysis. Similarly to the reaction catalyzed by HK and PFK, PK is an irreversible reaction. Thus, they are considered as the three control points of glycolysis pathway (Zanella et al., 2005; Sánchez-Paz et al 2008; Enes et al., 2009). In mammals the hepatic PK activity decrease during starvation periods or diabetes and increase as a response to high CBH dietary or insulin administration (Pilkis and Granner, 1992).

During low oxygen (hypoxia) events, the anaerobic glycolysis pathway takes place to produce energy. Lactate dehydrogenase (LDH), a key enzyme on this pathway, produces lactate from pyruvate in the last step of anaerobic glycolysis. It is reported that L. vannamei accumulates lactate and glucose on tissue (muscle and hemolymph) which are produced from pyruvate as the last step of anaerobic glycolysis (Racotta et al., 2002; Soñanez-Organis et al., 2012). Conversely, when there is no carbohydrate intake, there is the possibility of de novo production of glucose, named gluconeogenesis, from non-carbohydrate precursors, such as lactate, pyruvate, glycerol, and amino acids. Most crustaceans have some key enzymes of the gluconeogenesis pathway in the hepatopancreas (Cuzon et al. 2000; Cota-Ruiz et al., 2015), and the branchial tissue also contains enzymes involved in gluconeogenesis in common shore crab (Thabrew et al., 1971). The key enzymes involved in the gluconeogenesis are pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBPase) and glucose6-phosphatase (G6Pase). FBPase catalyzes the production of fructose 6-
phosphate from fructose 1,6-bisphosphate (Lozinska-Gabska et al., 2003; Kaiser et al., 1996). Rosas et al. (2001) have evidenced the activity of PEPCK in the hepatopancreas of whiteleg shrimp in response to the dietary composition (low protein and low CBH diet), strongly suggesting the utilization of the gluconeogenic pathway in crustaceans. In parallel with the glucose metabolism, the process of CBH transport and utilization are important and similar to that of mammals. Glucose is absorbed by hepatopancreas epithelial cells in the digestive gland then transferred into the hemolymph and carried throughout the organism to different organs via blood circulation (Menoyo et al., 2006; Wilson-O’Brien et al., 2010; Martínez-Quintana and Yepiz-Plascencia, 2012). The transport of glucose is mediated by the carrier proteins known as glucose transporters (Wilson-O’Brien et al., 2010). The glucose transporters can be classified into two super families in eukaryotic species, that is the Na⁺-dependent glucose transporters (SGLT) and the Na⁺-independent facilitative GLUT (Joost & Thorens, 2001; Wright, 2001; Hanquet et al., 2011). GLUT proteins utilize passive diffusion to transport glucose into the bloodstream. The GLUT protein can be expressed in any cell related to carbohydrate metabolism. In addition, the GLUT transporters are nearly expressed in all cells of the body to control glucose homeostasis.

Although 14 members of the GLUT family have been characterized in mammals with different transport kinetics, tissue distribution and regulatory properties (Zhao and Keating, 2007), in crustaceans, few studies have been done on the glucose transporters, only GLUT1; 2 and 5 (Caccia et al., 2007; Obi et al., 2011; Soñanez-Quintana et al., 2012; 2014). Similarly as in mammals, the glucose transporters proteins in crustaceans also show specific function. The best-known glucose transporter is GLUT1 because of its wide tissue distribution and high affinity towards glucose belonging to class I (Joost & Thorens, 2001; Martínez-Quintana and Yepiz-Plascencia, 2012). The GLUT2 is a bidirectional glucose transporter with function in relation with the low glucose concentration (to balance the glucose concentration between two compartments), showing low affinity for glucose (Zhao and Keating, 2007; Augustin, 2010; Soñanez-Quintana et al., 2012). GLUT2 is mainly expressed in hepatopancreas and intestine in vertebrates.
1.4.2.3 Lipid metabolism

The pathways involved on the lipid metabolism of aquatic species include lipid digestion and uptake, transport, synthesis (lipogenesis) and β-oxidation of fatty acids, as in mammals (NRC, 2011). As explained in the previous chapter, digestion of dietary lipids occurs in the pyloric region of the stomach. The hepatopancreas is the major source of digestive enzymes, like the triacylglycerol lipases (TAG lipase) and phospholipases (O’Conner and Gilbert, 1968; NRC, 2011). However, it also plays a role as a lipid storage organ, mainly triacylglycerides (TAG) which are an important source of energy during fasting or increase of energy demand (Birnbaum, 2003). The first and key enzyme involved in TAG catabolism is lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) that breaks down triacylglycerol into free fatty acids and glycerol (NRC, 2011; Riviera-Perez et al., 2011). The hydrolysis of stored TAGs (fat) or those exogenous TAGs (from dietary lipids) are catabolized by lipases which are classified into two main groups, lysosomal and digestive lipases. The endogenous TAG stored as lipid droplets are hydrolyzed by lysosomal lipases while digestive lipases are responsible to hydrolyze TAG in food (Roussel et al., 1999; Miled et al., 2000).

Lipogenesis is a biosynthesis reaction (anabolism) involved in the synthesis of de novo lipids, using the carbon source (acetyl-CoA) formed in the mitochondria by the oxidative decarboxylation of pyruvate (carbon source) or the oxidative degradation of some amino acids (protein source). The key enzyme on lipogenic pathway is catalyzed by the cytosolic fatty acid synthase (FAS), which produces the saturated fatty acids (FA), mainly 16:0 (palmitic acid) and 18:0 (stearic acid). These two FA are then converted into monounsaturated fatty acids (MUFAs), such as 18:1n-9 and 16:1n-7 through the action of Δ9-desaturase (stearoyl-CoA desaturase, SCD). On the other hand, the ability to synthesize highly unsaturated fatty acids (HUFA) varies among aquatic animals (Sargent et al., 2002; NRC, 2011; Tocher, 2003).

Shrimp have a limited ability to synthesize de novo the n-6 and n-3 FA families, including the polyunsaturated fatty acids (PUFAs), such as linoleic (18:2n-6, LOA) and linolenic (18:3n-3, LNA) (Suprayudi et al., 2004). Similarly, shrimp also have a limited ability to elongate and desaturase PUFAs into HUFAs.
Figure 1.4.2.4. Electron transport chain.
1.4.2.4 Energy (mitochondrial) metabolism

The center of the energy metabolism (ATP production) in aerobic conditions is inside the mitochondria (Figure 1.4.2.4). The mitochondrial $F_0F_1$ ATP-synthase complex (ATPase) is an enzyme with dual role, catalyzes the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Pi), either in eukaryotes or bacteria (Boyer, 1997). The enzyme consists of two major components, a catalytic headpiece $F_1$ (driven by ATP hydrolysis), and the base-piece/stalk membrane $F_0$ (embedded in the mitochondrial membrane and driven by a proton gradient) (Walker et al. 1991; Leslie and Walker, 2000). The catalytic headpiece portion of the enzyme ($F_1$) is composed of five major subunits ($\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$), where the sub-complex $\alpha$ and $\beta$ are characterized by three alternating pairs of subunits $\alpha/\beta$ forming a sphere that functions as an ATPase by hydrolyzing ATP to ADP+Pi, and as an ATP-producer by synthesizing ATP from ADP+Pi (Futai et al., 1989; Lai-Zhang and Mueller, 2000). All groups are encoded by the nuclear genome, translated in the cytoplasm, and then imported into mitochondria as pre-proteins (Walker and Runswick, 1983; Breen, 1988).

The mitochondrial $F_0F_1$ ATP-synthase enzyme synthesizes 95% of the ATP molecules in cells, and its function is closely related to the presence of oxygen that takes electrons yielded by this electron transport chain (Alberts et al., 2008). This catalytic process is triggered by the electrochemical gradient of protons generated along the electron transport chain in the mitochondrion (Pedersen, 2007). Mitochondrial cytochrome c oxidase (COX) is essential for aerobic energy generation in the form of ATP by the ATPase and is included in the complex IV of the mitochondrial respiratory chain. The cytochrome c oxidase is one of the key enzymes in aerobic metabolism, playing a fundamental role in energy production of aerobic cells by catalyzing the reduction of di-oxygen ($O_2$) to water. It represents the last step of electron transport chain, the transfer of electrons from cytochrome c to molecular oxygen and couples the free energy of the reaction to phosphorylate ADP to ATP (Verkhovsky et al., 2006; Mayevsky and Rogatsky 2007; Timón-Goméz et al., 2017). Many different COX proteins exist and are coded by either the mitochondrial or the nuclear DNA. The subunits of the enzymes COX I, COX II and COX III, are encoded in the mitochondrial genome of most species (Shen et al., 2007). The subunits encoded in the nucleus consist on a larger number, varying in number according with the species (Khalimonchuk and Rödel, 2005); including the COX VI (used in this thesis). All the nuclear subunits are cytoplasmically translated. According to Boone et al. (1993), the mammals COX subunits are characterized either as non-tissue-specific or tissue specific expression subunits, such as COX VIa L (liver type) and COX VIa H (heart/muscle type).
1.5 Nutritional Programming – searching for new feeding strategies

Modern shrimp farming relies on the delivery of industrially-manufactured aquafeeds. Feeds can account for up to 60% of production costs, driven primarily by the high dietary protein requirement of crustaceans. Several alternative ingredients are used to produce aquafeeds, but in comparison to fish meal, alternative proteins lack the same nutrient value and bioavailability. There are several reports of deleterious effects resulting from the replacement of fish meal protein for land-based plant proteins. The objective of this thesis is to improve the use of novel diets containing low protein levels and a high dietary inclusion of plant ingredients, through the evaluation of nutritional programming as a new feeding approach.

1.5.1 Concept of Nutritional programming

The concept of “programming” was reported for the first time by Lucas (1991). It refers to a long-term molecular or/and physiological adaptation of environmental factors (e.g., nutritional, pH, dissolved oxygen, etc.) through programming on critical (sensitive) phases of the early animal development (Lucas, 1998). The extension of this concept to the field of early nutrition is known as “nutritional programming” and has been largely studied in mammalian models to understand the consequences in adulthood of an altered nutrition during the intrauterine or post-natal periods (Burdge and Lillycrop, 2010; Devaskar and Thamotharan, 2007; Lucas, 1998; Metges et al., 2014; Ozanne and Hales, 1999; Patel and Srinivasan, 2002; Patel et al., 2009).

During development, plasticity allows to generate different phenotypes from the same genotype (Gluckman and Hanson, 2004), as it is well known in honey bees (Kucharski et al., 2008). It has been suggested that this process of developmental plasticity utilizes early nutritional cues to prepare individual phenotypes to better match a certain future nutritional environment (Gluckman et al., 2005; Lillycrop and Burdge, 2012). The early development and neo-natal period time during which several tissues and organs are in formation, are characterized by their high plasticity at the molecular level (Gluckman et al., 2011; Patel and Srinivasan, 2002). These periods are considered as the preferential developmental windows during which the programming could be more efficient.

Possible biological mechanisms for “nutritional imprinting”, i.e., recalling an earlier nutritional event in adulthood in mammalian vertebrates), comprise adaptive changes on gene expression and/or cellular phenotypes (linked to epigenetic mechanisms), on nutrient-sensitive signaling pathways, and on adaptive clonal selection which may be transmitted to future adults or
offspring (Lucas, 1998; Waterland and Jirtle, 2003; Symonds et al., 2009; Lillycrop and Burdge, 2012; Gut and Verdin, 2013;).

In mammals, nutritional stimulus during pregnancy, such as i) Growth Restriction or intrauterine growth retardation (IUGR), and ii) low birth weight related with maternal undernutrition, can increase the risks for the onset of non-communicable diseases or metabolic syndrome (type II diabetes, hypertension and cardiovascular disease) on the adulthood with deregulation of glucose homeostasis and lipid metabolism (Duque-Guimarães and Ozanne, 2013; Gluckman et al., 2005; Hales and Barker, 2001; Ozanne and Hales, 1999; Patel et al., 2009; Spencer, 2012). Indeed, there is clear evidence of the persistence of the programmed phenotype in adults as well as the possibility of its transmission to the offsprings (transgenerational or multigenerational effects) (Lim and Brunet, 2013; Vickers, 2014).

The most famous example of the nutritional programming concept is with the Dutch Hunger Winter, a famine that occurred in the Netherlands during the winter of 1944. This event explains some of the observed associations (from epidemiological studies) between progenitor and offspring. From September 1944 to May 1945, the supply of food was controlled by the Germans. A forced caloric restriction was made with the daily energy intake, dropping from 1,800 to 400-800 kcal/day. People suffered from chronic hunger and diseases related with malnutrition. Epidemiological studies performed in the offspring of mothers who suffered with famine are divided by different groups: i) famine during the period of peri-conceptual and early pregnancy, up to first trimester; individuals who did not suffer from low birth weight compared to unexposed individuals; however, at adult phase, they exhibited an increase of the risk to cardiovascular diseases, obesity and reduced cognitive functions; ii) famine during the middle gestational maternal famine; maternal undernutrition were associated with impaired kidney and lung function; iii) – famine during later pregnancy, individuals had reduced birth weights and at the adult phase shown increased incidence of insulin resistance at adult age, (Jiménez-Chillarón et al., 2012, Lillycrop and Burdge, 2012).

Several studies performed, mainly with mammals, demonstrate the long-term effect of early nutritional events on the metabolism. The exposure to an increasing range of insulin levels during the gestational period of rats, induced glucose intolerance in the offspring (Harder et al., 1998), while restricted intake of protein during the fetal or/suckling periods develop insulin resistance for the progeny on the late life (males at 15 months and females at 21 months) suggesting to be aged-dependent (Ozanne et al., 2003; Fernandez-Twinn et al., 2005). Other deleterious factors are also related with the early malnutrition (Srinivasan et al., 2008),
such as shorter lifespan (Aihie-Sayer et al., 2001), deregulation of glucose homeostasis (Fernandez-Twinn et al., 2005), vascular dysfunction (Torrens et al., 2006), increased susceptibility to oxidative injury (Langley-Evans and Sculley, 2005), impaired immunity (Calder and Yaqoob, 2000), altered feeding behavior (Bellinger et al., 2004) and increased central fat deposition (Bellinger et al., 2006). The use of an artificially high-carbohydrate milk formula during the lactation of rat pups before weaning resulted in an increased activity of the hepatic GK (Patel and Hiremagalur, 1992) and in altered mRNA transcription levels of genes involved in energy metabolism and appetite control that persisted into adulthood (Srinivasan et al., 2008).

The mechanisms involved in the regulation by early nutritional events are still not fully understood. However, studies have demonstrated strong evidence that the genome can be modulated and use a genetic memory as a result of nutritional event status (nutritional history). The epigenetic modifications are related with the imprinting genome. Epigenetics can be defined as the somatically heritable states of gene expression resulting from changes in chromatin structure without alterations in the DNA sequence. The principal epigenetic processes are DNA methylation, histone modifications, chromatin remodeling and non-coding RNAs (Choi and Friso, 2010, Jiménez-Chillarón et al., 2012, Lillycrop and Burdge, 2012). Furthermore, epigenetic changes are known to be possibly transmitted hereditarily, which largely supports the evidence of vertical transmission of metabolic-related diseases, from mothers to their progeny (Jaenisch and Bird, 2003; Youngson and Whitelaw, 2008). In aquatic species, the environment that fish experience in their early life lead to observable changes in their size, growth rate, metabolism and age at reproduction, among other aspects (Pittman et al., 2013). Similar to what is observed in mammals, the developmental windows with highest sensitivity and metabolic plasticity in fish are restricted to the periods of embryogenesis and early larval development (Geurden et al., 2007; Mennigen et al., 2013). Recent studies have demonstrated the effect of early nutritional programming is mainly related to the first-feeding. Indeed, the first study involving the nutritional programming in fish was performed by Geurden et al. (2007). An early nutritional stimulus, carried out by an acute hyperglucidic (60%) diet, exerted on the first-feeding stage in rainbow trout, induced the expression of the genes that coded to enzyme involved on the carbohydrate digestion in the juveniles, suggesting long-term physiology modifications. Data showed persistent molecular adaptations to the early nutritional history without differences on the growth performance and postprandial glycemia. Since then, other studies were performed using this approach, either related with the maternal nutrition (Izquierdo et al., 2015; Turkmen et al., 2017) or with the first stages of development, with
**Figure 1.5.2-1.** Possible examples of stimuli for nutritional programming in shrimp based on the concept of “metabolic programming” for mammals and fish.

**Figure 1.5.2-2.** The concept of nutritional programming (Lucas, 1998) and stimulus (feed restriction) used on this thesis.
different species, e.g., *Danio rerio* (Fang et al., 2014; Rocha et al., 2014, 2015), *Oncorhynchus mykiss* (Geurden et al., 2014; Liu et al., 2017; Panserat et al., 2017), *Salmo salar* (Vera et al., 2017), *Sparus aurata* (Rocha et al., 2016). A better acceptance of plant-based diets during the adult phase when alevins were fed with diets rich on vegetable content for weeks was also observed (Geurden et al., 2013). Thus, periods to exert a nutritional stimulus during stages of high developmental plasticity are narrowed to: i) maternal nutrition, through nutrient transfer to the yolk reserves and/or modifications of gamete epigenetics, and; ii) the onset of exogenous feeding. To our knowledge, no study on nutritional programming had been performed with the whiteleg shrimp, *L. vannamei* or other crustaceans. Based on the knowledge of this approach on fish, as explained in Figure 1.5.2-1, studies were conducted to investigate the concept of nutritional programming in juvenile *L. vannamei*, mainly by manipulating feed intake through feed restriction stimuli (Figure 1.5.2-2). The whiteleg shrimp, as a crustacean, goes through a typical metamorphosis process during early development, from embryo to nauplii, protozoea, mysis, and post-larvae, before it becomes an adult (Figure 3.1.1; Dall et al., 1990). Thus, nutrition manipulation during the first-feeding stages, is the best alternative for nutritional programming on this species.

### 1.5.2 Mechanisms of epigenetics: focus on aquaculture species

Conrad Waddington was the first credited using the term epigenetic in the early 1940s, as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being” (Waddington, 1968). Over the following years, the meaning of the word was gradually modified; today, epigenetic is generally defined as “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence” (Wu and Morris, 2001; Deans and Maggret, 2015). Based on this definition, epigenetic regulation at the molecular level is mediated by three main mechanisms, *i.e.*, DNA methylation, histones modifications (both mechanisms, regulate gene expression at the levels of chromatin structure and DNA), and the microRNAs modifications, a non-coding RNA regulating the abundance and translation of specific mRNAs.

These mechanisms can be affected by either environmental, endogenous signals or both, to regulate the genome-wide gene expression which leads to the emergence of physiological phenotypes (Best et al., 2018), such as those related to the mammal nutrition that are associated to DNA methylation (Weaver et al., 2004; Dolinoy et al., 2006). In fish, DNA methylation pattern
show sensitivity to the environment (Wang et al., 2009; Campos et al., 2013). The epigenetic marks induced by environmental issues can be reversible or may have persistent effect over the lifetime. Indeed, epigenetic response do not represent a permanent redirection of phenotype in the same way as does mutation or natural selection (Burggren and Crews, 2014; Dolinoy et al., 2006; Heijmans et al., 2008; Weaver et al., 2004).

It is important to consider the role of epigenetics to improve traits in aquaculture through the possibility to develop environmentally-driven phenotypes, achieved by sensitivess developmental stages or broodstock manipulation. Nutrition is at the origin of epigenetics modifications, playing an important role on mediating different phenotypes (Pittman et al., 2013; Metzger and Schulte, 2016; Turkmen et al., 2017).

Among the epigenetic mechanisms, the DNA methylation is the most widely studied (Deans and Maggert, 2015; Gavery and Roberts, 2017) and could be highly sensitive to nutritional parameters (Weaver et al., 2004; Turkmen et al., 2017). In this thesis, only the DNA methylation as epigenetic marks was analyzed. This is the reason why the following chapter only described the DNA methylation as an epigenetic mark.

1.5.2.1 DNA methylation and gene expression

Enzymes involved in DNA methylation act to methylate cytosines, through addition of a methyl group (CH₃) to the position 5 of the pyrimidine ring of a cytosine (5mC). These marks are mostly, but not exclusively, located in the context of genomic CpG sites (CpG dinucleotides; cytosine-phosphate-guanine) for the eukaryotic cells (Ramsahoye et al., 2000). Methylated cytosines can also occur on non-CpG sites (CpHpG and CpHpH), and are characterized as strand-specific epigenetic mark, differently from CpG methylation (Law and Jacobsen, 2010). Such DNA methylation, through the addition or removal of methyl groups, can subsequently modify gene transition; indeed, methylation of GpC sequences at transcription start sites (TSS) has been associated with down expression of gene (Jones, 2012; Li and Zhang, 2014, Best et al., 2018).

1.5.2.2 Main mechanisms involved in DNA methylation

The enzymes that catalyze DNA methylation are named as DNA methyltransferases (DNMTs), which are divided into DNMT1 and DNMT3 families. In mammals, the maintenance of
methylated DNA bases during the mitotic inheritance is assured by DNMT1 (Leonhardt et al., 1992; Robertson and Jones, 2000). By contrast, the DNMT3 family is divided into DNMT3A and DNMT3B. These enzymes are mainly responsible for the de novo methylation of unmethylated sites (Cheng, 2014; Okano, et al., 1999). The global DNA methylation levels in fish are higher than those in mammals, although the significance of this difference remains unclear (Zhang et al., 2016). However, the pattern on the DNA methylation in invertebrates is different from the vertebrate one, as observed in vertebrates which exhibit a global pattern of DNA methylation. Invertebrates show a ‘mosaic’ pattern with stretches of methylated DNA punctuating regions of unmethylated DNA (Bird and Taggart, 1980; Tweedie et al., 1997; Feng et al., 2010; Gavery and Roberts, 2017). For example, Pestana et al. (2016) reported a very low level of the DNA methylation of brine shrimp Artemia franciscana (crustacean animal model) exposed to heavy metals.

While DNA methylation marks in the genome occurs through an active process, the de-methylation can either passively through cell division and absence of maintenance of DNA methylation, or actively via enzymatic pathway, which catalyze the removal of DNA methylation (Wu and Zhang, 2010, Bhutani et al., 2011). Active de-methylation is mediated by 5-methylcytosine (5mC) oxidation catalyzed by members of the ten-eleven translocation (TET) methylcytosine dioxygenase family, yielding 5-hydroxymethylcytosine (5hmC) and subsequently 5-formyl-cytosine and 5-carboxylcytosine (Xu and Wong, 2015; Kamstra et al., 2015).
Chapter 2

Thesis objectives
This thesis focused on the evaluation of a novel feeding strategy called nutritional programming in juveniles of the whiteleg shrimp, *L. vannamei*. Studies were designed to investigate the impact of early nutritional stimulus (feed restriction) on the use of nutrients in shrimp juveniles. Based on growing evidence, nutritional programming in animals is effective from invertebrates (such as bees) up to humans. Nutrition, molecular biology and biochemistry were used to investigate the differences in intermediary metabolism. The work covered three specific objectives:

I – Determine the ontogenesis of many of the genes involved in intermediary metabolism in shrimp in order to choose the best developmental window for the nutritional programming (paper I “Ontogenesis of metabolic gene expression in whiteleg shrimp (*Litopenaeus vannamei*): New molecular tools for programming in the future.”)

II – Investigate the effect of early feed restriction (at 70%) at the post-larval stage and analyze long-term metabolic and nutritional consequences in shrimp juveniles fed with novel diets (paper II “Metabolic programming in juveniles of the whiteleg shrimp (*Litopenaeus vannamei*) followed by an early feed restriction at post-larval stage.”).

III – Investigate the effect of early feed restriction (at 40%) at the protozoa stage and analyze long-term metabolic and nutritional consequences in shrimp post-larvae (paper III “Four-day feed restriction at the protozoea stage showed little effect on long-term metabolic gene expression in whiteleg shrimp, *Litopenaeus vannamei*”).
Chapter 3

Material and methods
3.1 The ontogenic study

Samplings of *L. vannamei* larvae were performed on a commercial hatchery production (CELM Aquicultura S/A) located in the city of Aracati, 160 km distant from Fortaleza capital, NE Brazil. During larval production, female broodstock of the whiteleg shrimp spawned together in indoor round tanks and floating eggs were collected and transferred to hatching tanks. In the following day, healthy nauplii were collected with the assistance of an artificial light (positive phototropism), rinsed with sterile seawater, disinfected (formalin or iodine), then rinsed again and stocked in 50 L recipients to estimate population size. Finally, as animals metamorphize into nauplii N3 (nauplius substage 3), they were stocked under 400 animals/L and reared until post-larvae 10 (PL10) or PL12, in a single-phase static outdoor tank system. Dissolved oxygen, pH, temperature, and salinity were monitored daily. Total ammonia nitrogen, nitrite, nitrate and phosphate were monitored daily through colorimetry. At the zoea stage, larvae were fed with a mix of microalgae (*Thalassiosira* spp and *Chaetoceros* spp) and multiform of commercial larval diets. During mysis substages and post-larvae, shrimp were fed a mix of hatched *Artemia* cysts and commercial larval diet.

3.1.1 Life cycle identification and sampling

A description of shrimp larval cycle, from hatching until PL10 stage, is provided in Table 1.2.1 and Figure 3.1.1. Based on this information, sampling was performed on all shrimp developmental stages, from spawning to PL10. Thus, sampling was carried out as follows: nauplii in substages N1, N3, and N5; protozoea substages Z1, Z2, and Z3; mysis substages M1, M2, and M3; and, post-larvae substages PL1 through PL10 (Wei *et al.*, 2014ab). Samplings of embryo stages were collected based on time, i.e., hours after of spawn, such as, 0, 2, 4, and 8 h after spawning.

Gene expression analyses were performed on the stages that compromise main metamorphoses over the whole shrimp larval development. A total 10 different developmental stages were analyzed, with the first sampling taking place at zygote phase, 8 h after spawning (E 8h). Subsequent to post hatching, shrimp were collected during nauplii, sub-stage N5; zoea, sub-stages Z1, Z2, and Z3; mysis, sub-stages M1, M2, and M3; and; two post-larval sub-stages, PL1 and PL5.

For sampling, shrimp larvae were washed with sterile seawater and dried with an absorbent paper. Samples were preserved on an RNA Stabilization Solution (RNA Later Sigma®), 100 mg
Figure 3.1.1. Embryo and larval stages during early development of *L. vannamei*. Adapted from Wei *et al.*, (2014a). N: Nauplii; Z: Protozoa; M: Mysis; P: Post-larvae
tissue /1 mL RNALater. To ensure that the RNA solution permeated through the samples, the mix buffer and the sample were kept for 24 at 4°C, then stored at –20°C. This protocol was adopted to all the samples used for gene expression analysis. Before sampling, visual evaluations under a microscopy were performed to ensure at least 90% of the individuals at sampling corresponded to the desired developmental stage (Treece and Fox, 1995; Wickins and Lee, 2002; Juarez and Moss, 2010; Wei et al., 2014a).

3.1.2 Primers design for analysis of mRNA levels

Twenty genes (mRNA levels) coding for digestion and intermediary metabolism were studied using specific primers for shrimp. Some were previously described for the genes coding for digestive enzymes (lipase, preamylase, trypsin and chymotrypsin) (Wei et al., 2014b). The new primers design was performed using the Primer 3 software. Specific primer pairs were designed with an overlapping intron, when possible, using known L. vannamei sequences in nucleotide GeneBank databases. In order confirm their specificity, amplicons obtained with the designed primer were purified and sequenced (GENEWIZ Beckman Coulter Genomics, UK). Database accession numbers and the sequences of forward and reverse primers used to test each gene are provided in Table 3.1.2. The choice of the genes was detailed in the introduction chapter.

The protocol for analysis of mRNA levels is detailed below, and it is also similar to all, including the two programming studies experiments.

3.1.3 RNA extraction

Biological material was collected with a sterile mesh (100 µm) and then dried with an absorbent paper to remove the maximum amount possible of RNA Later. Subsequently, the tissue was weighed and immediately immersed on a Trizol solution. RNA extraction from whole body (pool of animals) or tissue (hepatopancreas and muscle; one sample per animal) was performed using the reagent Trizol® (100 mg sample / 1 mL Trizol). The concentration was determined through the spectrophotometer NanoDrop 2000 and the quality determined by 1% agarose gel electrophoresis.
<table>
<thead>
<tr>
<th>Function</th>
<th>Reference Gene</th>
<th>Genes</th>
<th>Primers 5’-3’</th>
<th>Access number</th>
</tr>
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<tr>
<td><strong>Luciferase</strong></td>
<td></td>
<td>Luciferase Control RNA (Promega, L4561)</td>
<td>cattttctgcaaaaaaagcactctg agccccatctcttgctgtacccc cttccgactcttgaagacgc</td>
<td>(cf Marandel et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>ef1a</td>
<td><em>Litopenaeus vannamei</em> elongation factor 1-alpha mRNA</td>
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<tr>
<td></td>
<td>hk</td>
<td><em>Litopenaeus vannamei</em> hexokinase mRNA</td>
<td></td>
<td>EF102106</td>
</tr>
<tr>
<td></td>
<td>pk</td>
<td><em>Litopenaeus vannamei</em> pyruvate kinase mRNA</td>
<td></td>
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<tr>
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<td>ldh</td>
<td><em>Litopenaeus vannamei</em> lactate dehydrogenase mRNA</td>
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<td></td>
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<td>KP057246</td>
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<td></td>
<td>pepck</td>
<td><em>Litopenaeus vannamei</em> phosphoenolpyruvate carboxykinase gene</td>
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<td>chh</td>
<td><em>Litopenaeus vannamei</em> crustacean hyperglycemic hormone B (CHH-B) gene</td>
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<tr>
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<td>lvglut 1</td>
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<td>fas</td>
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<tr>
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<td></td>
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<tr>
<td></td>
<td>gs</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Energy Metabolism</strong></td>
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<td><em>Litopenaeus vannamei</em> mitochondrial cytochrome C oxidase subunit V1a precursor, mRNA</td>
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<td>KF906252</td>
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<tr>
<td></td>
<td>cox VI a</td>
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<tr>
<td></td>
<td>cox VI b</td>
<td></td>
<td></td>
<td>KF906253</td>
</tr>
</tbody>
</table>
**cox VI c**  
*Litopenaeus vannamei* mitochondrial cytochrome C oxidase subunit VIc mRNA

**atpase a**  
*Litopenaeus vannamei* mitochondrial ATP synthase subunit alpha precursor, mRNA

**atpase b**  
*Litopenaeus vannamei* mitochondrial ATP synthase subunit beta precursor, mRNA

**Digestion**

**lipase**  
Triacylglycerol lipase

**preamylose**  
Preamylase 1

**trypsin**  
Trypsin

**chymotrypsin**  
Chymotrypsin BII

(cf Wei et al., 2014b)
3.1.4 Quantification of target genes by qRT-PCR

Gene expression levels were determined by real-time RT-PCR (n=5; RNA sample per stage of the development; whole body). Twenty genes (mRNA levels) were studied using specific primers. An amount of 1µg RNA was reverse transcribed to cDNA with SuperScript III RNaseH-Reverse Transcriptase Kit (Invitrogen) with random primers (Promega). Real-time PCR was performed in the LightCycler 480 (ROCHE, Hercules, CA, USA). Quantitative PCR (Q-PCR) analyses for gene expressions were performed using a reaction mix of 6 µL per sample containing 2 µL of the RT produce (diluted cDNA), 0.24 µL of each primer (10 µmol/L), 3 µL Light Cycler 480 SYBR® Green and 0.54 µL DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany) as previously described (Dai et al., 2014). Melting curves were systematically monitored (temperature gradient at 0.5 °C/10 s from 55 to 94 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction (Figure 3.1.4). Each q-PCR run included duplicates of samples (reverse transcription) and negative controls (wells without reverse transcriptase, mRNA and cDNA). Relative quantification of target gene expression was performed using the ΔCT method (Pfaffl, 2001). The reference genes L. vannamei elongation factor 1-alpha (ef1a) or Luciferase (exogenous Promega®) were used for the normalization (data not shown). In all cases, PCR efficiency (E) was measured by the slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency values ranged between 1.8 and 2.2.
Figure 3.1.4. Representation of the RT-qPCR. A: Amplification curve; number of cycles necessary to produce the maximum number of DNA double band, which emits a fluorescence signal though the presence of SYBR Green reactive; B: Melting peak, specificity of amplification reaction (oligonucleotides).

Figure 3.2 – 1. Experimental rearing tanks used for the hatchery and nutritional programming at shrimp protozoea phase. Laboratory of Aquatic Animal Nutrition LANOA – LABOMAR/UFC (Eusébio, Brazil).
3.2 II – Characterization of experimental units for the programming studies

For a better understanding, this section describes the different experimental units used in all assays. Seawater was pumped into two 20 m³ reservoirs located at LABOMAR’s experimental aquaculture facility (Eusébio, Brazil). Water was then disinfected with chlorine (0.02 g/L), and sand-filtered for 24 h to remove large particles. Finally, water was transferred to shrimp rearing tanks used in this study.

- Rearing system for the Protozoea experiment

The system was characterized by 44 rectangular tanks of 61 L in volume, measuring 31.0 x 35.5 x 55.5 cm (height x width x length). The system operating on a clear water with continuous water recirculation and filtration using a sand filter (particles larger than 50 µm). The system was connected to two reservoir tanks of 10 and 5 m³ for storage of filtered and disinfected seawater. In all rearing tanks, there was a continuous aeration provided by two 2.0-hp air blowers and one air diffuser per tank (Figure 3.2 - 1). Only 16 experimental tanks were used in this study.

Seawater was transferred to two reservoirs (10 and 5 m³) to adjust salinity to 30 ups, disinfected with chloride, and sand filtered for 48 h. To assure there was no remaining chlorine, a chlorine test (Labcon CloroTest, Alcon) was performed. Finally, sodium thiosulphate (5 ppm) was added as a chlorine neutralizer.

During the experimental study, culture water was kept static, without any water recirculation. Whenever necessary, the tank bottom was siphoned to remove sludge. A 12-h artificial light cycle was setup during the complete study period. Shrimp were raised from nauplii to PL stage in this rearing system.
Figure 3.2 – 2. Overview of 40 experimental tanks of 1,500 L operated on green-water used for the nutritional programming at post-larval phase. Laboratory of Aquatic Animal Nutrition LANOA – LABOMAR/UFC, Eusébio, Brazil.
• *Rearing system for the post-larvae experiment*

For this study, a new outdoor rearing system was built with financial support from Neovia (Figure 3.2 – 2).

The system consisted of 40 polypropylene round tanks of 1,500 L in volume (bottom surface area 1.60 m²) operated under green-water and minimum water exchange. Tanks were kept outdoors, exposed to a natural photoperiod (12 h light: 12 h dark) and weather changes. Continuous water aeration was provided by two 4-hp blowers and an air diffusing system made with a 1-m aeration tubing (Aero-Tube™, Tekni-Plex Aeration, Austin, Texas, USA) rested near the bottom of each tank. Water was aerated continuously to reach near dissolved oxygen saturation (above 5 mg/L).

Tanks were covered with a perforated lid to avoid shrimp escape and high light intensity and water temperature. Rearing tanks were connected with four water reservoirs of 20 m³ tanks to supply sand-filtered seawater.

**3.3 III – Nutritional programming study at the protozoea phase**

The effect of a moderate feed restriction (40% reduction of total feed allowance) was evaluated during the protozoea stage. A total of 120,000 nauplii at sub-stage 3 of *L. vannamei* were purchased from a commercial hatchery (CELM Aquicultura, Aracati, Ceará, Brazil) and transported in by road to the LABOMAR’s aquaculture research facilities in double plastic bags containing seawater saturated with dissolved oxygen. A total of 7,500 nauplii at substage N3 were stocked at night in 16 indoor tanks of 61 L under 125 naupli/L. A four-day stimulus was initiated when shrimp metamorphosed from nauplii to protozoea.
Figure 3.3.1. Overview of the experimental design from the early nutritional stimulus at the protozoea stage up to juvenile stage. All sampling was carried out as a pool of the whole shrimp body. §: evaluation of growth performance.

Figure 3.2.2. Production of the microalgae *Thalassiosira* spp. This microalgae was used as live feed from the late naupli N5/early protozoea Z1 sub-stages to post-larvae PL3.
3.3.1 Experimental design

The nutritional programming scheme used for protozoa is described in Figure 3.3.1. Animals were exposed to two experimental conditions, a normal feeding protocol (CTL; BernAqua NV, Olen, Belgium) and a feed-restricted group (RES), where 16 rectangular tanks of 61 L were used as experimental units, totalling eight replicates for each experimental group.

3.3.2 Stimulus protocol through feed restriction

The early nutritional stimulus started when larvae reached the protozoa Z1 sub-stage 1. Animals were then divided into one experimental group (RES) and a control group (CTL). Protozoa shrimp were fed following a commercial hatchery feeding guide (BernAqua NV, Olen, Belgium). The CTL groups were fed following normal feeding rates while RES was raised under a 40% restriction in feed allowance (Table 3.3.2). The volume of microalgae delivered to the feed-restricted group was decreased in accordance to the feeding protocol. Thus, sterile salt water was added in the RES group tanks to maintain a similar water level.

During the experiment, no disinfectants or probiotics were used. The microalgae *Thalassiosira* spp was used as live feed from the late naupli N5/early protozoa Z1 sub-stages to post-larvae PL3. *Artemia* cysts were completely replaced by Vitellus (BernAqua). Chlorine (20 ppm) was utilized to sterilize seawater. Afterwards, chlorine was neutralized with sodium thiosulphate at 5 ppm. Microalgae was produced at LANOA on a batch outdoor production. A new stock was inoculated daily to a medium cell culture Gillard F/2 (Lavens and Sorgeloos, 1996). A microalgae stock culture was maintained on a cold room with diffused luminosity, and new batches were initiated according with the demand. Every two days, the microalgae volume was duplicated with sterile seawater (chlorine at 20 ppm for 24 h, then neutralized with sodium thiosulphate at 5 ppm, followed by verification of the presence of chlorine with a chlorine test) added medium Guillard F/2 (Figure 3.3.2).

After the early stimulus (4 days) all the experimental groups were fed similarly, following the BernAqua feeding protocol, until the end of the experiment at the 40th day of rearing.
Table 3.3.2. Stimulus Restriction Feeding Protocol for *L. vannamei* (during 4 days from protozoea Z1 sub-stage up to the Z3 sub-stage). CTL: control group. RES: feed-restricted group (40% of reduction of the feed allowance of the CTL group).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>Microalgae²</th>
<th>Royal Caviar³</th>
<th>Vitellus Standard ⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>1</td>
<td>80</td>
<td>10.88</td>
<td></td>
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<tr>
<td></td>
<td>2</td>
<td>80</td>
<td>14.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>100</td>
<td>18.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>120</td>
<td>22.28</td>
<td>121.50</td>
</tr>
<tr>
<td>RES</td>
<td>1</td>
<td>48</td>
<td>6.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>48</td>
<td>8.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>60</td>
<td>11.21</td>
<td></td>
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<tr>
<td></td>
<td>4</td>
<td>72</td>
<td>13.70</td>
<td>72.90</td>
</tr>
</tbody>
</table>

Proximate composition (%, as is)⁵

<table>
<thead>
<tr>
<th></th>
<th>Proteins</th>
<th>Lipids</th>
<th>Ash</th>
<th>Fiber</th>
<th>Nitrogen Free Extract ⁵</th>
<th>Moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>55.0</td>
<td>10.0</td>
<td>12.0</td>
<td>1.7</td>
<td>13.3</td>
<td>8.0</td>
</tr>
<tr>
<td>RES</td>
<td>51.0</td>
<td>10.0</td>
<td>10.0</td>
<td>3.5</td>
<td>17.5</td>
<td>8.0</td>
</tr>
</tbody>
</table>

³Feeding guide based on BernAqua NV (Olen, Belgium) recommendations.

²*Thalassiosira* spp (1000 cel/mL).

³(BernAqua NV, Olen, Belgium). Microcapsulated larval diet. Particle size from 50-100 µm.

⁴(BernAqua NV, Olen, Belgium). Particle size from 50-125 µm.

⁵Product label. Manufacturer guarantee nutrient levels.

⁶NFE, calculated by difference (100 – crude protein – crude fiber – crude fat – ash).
3.3.3 Sampling

Shrimp larvae were sampled three times after the early stimulus (Day 5), at the end of hatchery phase (Day 25) and at harvest (Day 40) (Figure 3.3.1). For all samplings, the pool of shrimp larvae was washed with sterile salt water, and dried with an absorbent paper. Samples were preserved on a RNA Stabilization Solution (RNA Later Sigma®), 100 mg tissue /1 mL RNA Later, immediately immersed on this solution and kept under −20°C.

3.4 IV – Nutritional programming at the post-larvae phase

The nutritional programing concept was evaluated on the early phase of post-larval *L. vannamei*. At post-larvae stage 1 (PL1), shrimp were subjected to a severe feed restriction (70% of feed allowance reduction) during three days. A total of 30,000 PL 1 of *L. vannamei* were brought to the lab from commercial hatchery (Potiporã Aquacultura Ltda., Beberibe, Brazil) in plastic bags containing seawater saturated with dissolved oxygen (DO).

3.4.1 Experimental design

The nutritional stimulus started when the hatchery and nursery (up to juvenile 1.0 g) phases ended. It was performed during three days using six 3,000 L circular tanks. Animals were divided into one experimental group and a control (CTL) group with three replicate tanks each. Following the nutritional stimulus, shrimp were fed similarly until the 50th day when juveniles were transferred to 1,500 L circular tanks. In these tanks, the diet challenge phase was carried out. Both CTL and RES groups were challenged for a 70-day period with three different diets in 30 tanks (3 dietary treatments x 2 groups x 5 replicates). The experimental design is summarized above (Figure 3.4.1).

Seawater transferred from 20 m³ had the salinity adjusted to (28 ups) adding fresh water and disinfected with chlorine (0.02 g L⁻¹). Subsequently, water was sand-filtered for 24 h to remove large particles, filtered by a carbon filter, then used to fill six circular fiberglass tanks of 3 m³. Water of each tank was fertilized with 100 g of a grounded commercial starter shrimp feed and 200 g of dried sugar-cane molasses. Strong aeration was supplied through mechanical blowers to provide sufficient dissolved oxygen (DO) and water circulation for plankton growth.
Figure 3.4.1. Schematic overview of the feed restriction stimulus up to the dietary challenge in juvenile shrimp. All the different sampling are presented: whole body were collected at the 1st and 2nd samplings; hepatopancreas and muscle were extracted at the 3rd and 4th samplings.
3.4.2 Stimulus and hatchery

In the laboratory, PLs (1.10 ± 0.10 mg body weight) were stocked equally in each rearing tank under 1.6 PL/L. For the early nutritional stimulus, PLs of 1.10 ± 0.10 mg body weight were divided into one experimental group and a control (CTL) group, assigning three replicate tanks for each. Post-larval shrimp were fed for three days, following a commercial hatchery feeding guide (BernAqua NV, Olen, Belgium). The CTL group were fed under a regular feed allowance while the feed-restricted treatment (RES) were subjected to a 70% feed reduction (Table 3.4.2). During the stimulus period, no live feed (*Artemia* spp. and microalgae) was delivered to PLs, although it could be naturally available in water. Additionally, tanks were shaded to avoid excessive growth of phytoplankton which could interfere with the nutritional stimulus. Thus, feed restriction was based solely on limiting allowance of inert food. In the end of the stimulus phase, juvenile shrimp were reared during an additional period of 47 days before transferring them to tanks where the challenge phase was carried out.

3.4.3 Diet production

The diets used on this thesis for the final nutritional challenge were formulated in agreements with all the partners (Labomar, INRA and Neovia). It is important to highlight the interest of Neovia company to develop new diets for aquaculture. Initially, a nutrition study was performed to validate diets with high replacement of fish meal by plant protein and carbohydrates (to reduce the protein content). This served later as a basis for the nutritional programming study (final nutritional challenge), as explained below.

For this study, diets were designed using the formulation software Optimal Formula 2000 (Optimal Informática Ltda., Campinas, São Paulo, Brazil). First, a basal diet (P43) was formulated to contain 15.0% fishmeal (% of the diet, as is basis), 43.3% crude protein (CP, dry matter basis, DM) and a NFE (nitrogen-free extract) and CP ratio of 0.7. Afterwards, three other diets were designed by replacing fishmeal with wheat flour and soybean meal, so that CP was reduced to 36.9, 34.7 and 29.6% (diets P37, P35 and P30, respectively) with NFE/CP ratios of 1.0 and 1.5, respectively (Table 3.4.3).
Table 3.4.2. Feeding protocol (g of diet per day) used for stimulus of post-larval *L. vannamei* (between PL1 and PL4 stage). CTL: control group. RES: feed restricted group (70% restriction).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>Feeding Protocol (g/day, as is)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diet 1²</td>
</tr>
<tr>
<td>CTL</td>
<td>1</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.037</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.015</td>
</tr>
<tr>
<td>RES</td>
<td>1</td>
<td>0.288</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.311</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.305</td>
</tr>
</tbody>
</table>

Proximate composition (% as is)⁵

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>51.0</td>
<td>55.0</td>
<td>54.0</td>
</tr>
<tr>
<td>Lipids</td>
<td>10.0</td>
<td>10.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Ash</td>
<td>10.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Fiber</td>
<td>3.5</td>
<td>1.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Nitrogen Free Extract⁶</td>
<td>17.5</td>
<td>13.3</td>
<td>16.0</td>
</tr>
<tr>
<td>Moisture</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Ratio NFE: Crude protein</td>
<td>0.34</td>
<td>0.24</td>
<td>0.30</td>
</tr>
</tbody>
</table>

¹Feeding guide based on BernAqua NV (Olen, Belgium) recommendations.
²Vitellus Standard (BernAqua NV, Olen, Belgium). Particle size from 125-400 µm.
³Royal Caviar (BernAqua NV, Olen, Belgium). Microcapsulated larval diet. Particle size from 100-200 µm.
⁴Royal Seafood (BernAqua NV, Olen, Belgium). Special diet for post-larval stages. Particle sizes from 100-200 µm.
⁵Product label. Manufacturer guarantee nutrient levels.
⁶NFE, calculated by difference (100 – crude protein – crude fiber – crude fat – ash).
Initially four diets were formulated, produced and evaluated during the challenge phase (Figure 3.4.3). Data regarding for this diet is presented as complementary data.

Diets were manufactured with a laboratory extruder according with Nunes et al. (2011). Briefly, all grain ingredients were grounded to achieve a particle size lower than 600 microns and FM was sieved through a 225 microns mesh net. After grinding or sieving, all dry and liquid ingredients were weighed to a 0.01 on an electronic scale. The macro ingredients were mechanically mixed for 10 min with a baker blender, while micro nutrients were mixed with a “Y” blender for 10 min. at 30 RPM. Afterwards, all ingredients were mixed again for 5 min., when fish oil and soy lecithin were included mixed, and then boiled water added. The dough was mixed one last time, sieved on 5000 microns mesh and pelletized using a small-scale extruder with a 1.8 die at 90°C (EXTEEC Máquinas, Ribeirão Preto, São Paulo, Brazil). Finished feeds were transferred to a pot for 3 min for steam-cooking at 100°C, dried in a convection oven at 60°C for approximately 3 h until 8-10% moisture was reached. Feed moisture was continually evaluated during drying using a rapid moisture analyzer (MB35 Moisture Analyzer, Ohaus Corporation. New Jersey, USA).

Finally, diets were cooled under room temperature, packed in plastic bags and stored at –20°C. Proximate composition was carried out for each feed using standard methods (AOAC, 2002).
Table 3.4.3. Ingredient composition (% as is) and proximate composition (% DM basis) of experimental diets used during the dietary challenge.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>P30</th>
<th>P35</th>
<th>P37</th>
<th>P43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean meal</td>
<td>35.29</td>
<td>41.15</td>
<td>41.28</td>
<td>45.21</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>46.69</td>
<td>38.29</td>
<td>35.61</td>
<td>24.97</td>
</tr>
<tr>
<td>Salmon meal</td>
<td>3.00</td>
<td>7.00</td>
<td>11.00</td>
<td>15.00</td>
</tr>
<tr>
<td>Cassava starch</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.00</td>
</tr>
<tr>
<td>Salmon oil</td>
<td>3.14</td>
<td>3.08</td>
<td>3.03</td>
<td>3.02</td>
</tr>
<tr>
<td>Soy lecithin</td>
<td>3.73</td>
<td>3.44</td>
<td>3.15</td>
<td>2.85</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>1.75</td>
<td>1.56</td>
<td>1.36</td>
<td>1.20</td>
</tr>
<tr>
<td>Vitamin-mineral premix</td>
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<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Squid meal</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.67</td>
<td>0.67</td>
<td>0.67</td>
<td>0.66</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.45</td>
<td>0.37</td>
<td>0.30</td>
<td>0.23</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.24</td>
<td>0.26</td>
<td>0.28</td>
<td>0.31</td>
</tr>
<tr>
<td>Synthetic binder</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>2.46</td>
<td>1.62</td>
<td>0.78</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Cholesterol SF</td>
<td>0.05</td>
<td>0.03</td>
<td>0.02</td>
<td>-</td>
</tr>
</tbody>
</table>

Proximate composition (% DM)

<table>
<thead>
<tr>
<th>Composition</th>
<th>P30</th>
<th>P35</th>
<th>P37</th>
<th>P43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>29.55</td>
<td>34.70</td>
<td>36.91</td>
<td>43.27</td>
</tr>
<tr>
<td>Lipids</td>
<td>5.67</td>
<td>6.50</td>
<td>5.97</td>
<td>5.24</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>2.51</td>
<td>2.80</td>
<td>2.92</td>
<td>3.84</td>
</tr>
<tr>
<td>Ash</td>
<td>7.20</td>
<td>7.54</td>
<td>7.31</td>
<td>8.13</td>
</tr>
<tr>
<td>Nitrogen Free Extract</td>
<td>45.49</td>
<td>38.92</td>
<td>37.66</td>
<td>29.35</td>
</tr>
<tr>
<td>Gross energy</td>
<td>16.2</td>
<td>16.4</td>
<td>16.6</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Ratio NFE: Crude protein

1. Bunge Alimentos S.A. (Luiz Eduardo Magalhães, Brazil). 48.80% crude protein (CP), 1.30% lipids, 5.94% fiber, 6.14% ash, 10.30% moisture.

2. J. Macedo (Fortaleza, Brazil). 9.21% CP, 1.32% lipids, 0.21% fiber, 0.62% ash, 12.73% moisture.

3. Pesquera Pacific Star S.A. (Puerto Montt, Chile). 59.38% CP, 9.40% lipids, 6.50% fiber, 22.90% ash, 15.77% moisture.

4. 0.25% CP, 0.03% lipids, 0.70% fiber, 13.29% moisture.

5. Rovimix® Camarões Intensivo, DSM Produtos Nutricionais Brasil Ltda. (São Paulo, Brazil). See (Sá et al., 2013) for composition.

6. 83.13% CP, 5.65% lipids, 9.75% moisture.
Aquavi® Lys, Evonik Nutrition & Care GmbH (Hanau, Germany). 50.7% lysine.
ThreAMINO®, Evonik Nutrition & Care GmbH (Hanau, Germany). 98.5% threonine.
Nutri-Bind Aqua Veg Dry, Nutri-Ad International NV (Dendermonde, Belgium).
Serrana Foscálcio20, Bunge Fertilizantes S.A. (Cubatão, Brazil). 20.5% calcium, 20.2% total phosphorus.
Rovimix® Stay C® 35, DSM Produtos Nutricionais Brasil Ltda. (São Paulo, Brazil). 350.0 g kg⁻¹ phosphorylated vitamin C.
Cholesterol SF, Dishman Netherlands B.V. (The Netherlands).
Calculated by difference (100 – crude protein – crude fiber – crude fat – ash).
Estimated values given as MJ kg⁻¹.
Figure 3.4.3. Experimental diets.
3.4.4 Dietary challenge for juvenile shrimp

Animals weighing 0.9 ± 0.10 g (mean ± standard deviation; n = 1,680, CV = 11%) were stocked in outdoor tanks under 30 shrimp m⁻² and reared for 70 days (56 shrimp/tank). Prior to the dietary challenge, shrimp were acclimated for one week with a commercial grower shrimp feed (Density 38 J, Neovia Nutrição e Saúde Animal Ltda., São Lourenço da Mata, Pernambuco, Brazil). The rearing system was composed of 30 round tanks of 1,500 L (bottom surface area of 1.60 m²). Tanks operated under green-water conditions and a water exchange of 10% of total water volume was applied weekly. Water was aerated continuously to reach near dissolved oxygen saturation (above 5 mg/L). Water pH, temperature and salinity were monitored once daily in the afternoon in each tank. Shrimp were fed four times a day at 0700 am, 1000 am, 0100 pm, and 0400 pm, exclusively in feeding trays (Figure 3.4.4). Once a week, shrimp were sampled and weighed in order to adjust feed rations.

At harvest, shrimp were individually weighed to a 0.01-g electronic scale and counted to determine their final body weight (FBW, g), specific growth rate (percentage increase in size per day – SGR; %/day, given as SGR = [(lnFBW – lnIBW)/∆t (days)] x 100), final survival (%), gained yield (g of biomass gained/m²). Feed conversion ratio (FCR = AFI/Final gained biomass) was determined based on apparent feed intake. (AFI, g of total feed delivered per stocked shrimp). Daily, before each meal, all uneaten feed was collected and stocked under -20 °C. At the end of the experimental period, feed remains were oven-dried and weighed to calculate AFI (Façanha et al., 2016; Nunes et al., 2006b).

3.4.5 Samplings

Biological material (larvae whole body and tissue samples) were preserved on an RNA Stabilization Solution (RNA Later, Sigma®) and kept stored under -20°C. Juvenile shrimp sampled were individually weighed before samplings tissues. Firstly, the hemolymph was withdrawn, then shrimp were excised to extract their hepatopancreas and caudal muscle; respectively. Figure 3.4.1 shows the different period for sampling over the study period.
Figure 3.4.4. Manual feeding allowance using a PVC feed tray, 200 mm diameter.

Figure 3.4.5.1 Illustration of hemolymph withdraw. Source: Patricia Vieira.
3.4.5.1 Hemolymph and plasma sampling

At the end of challenge phase (4th sampling, Figure 3.4.1), hemolymph sampling was performed 3 h after the last meal, based on a study performed with *Penaeus monodon* (Richard, 2010; data unpublished) which is equivalent to the species postprandial peak. Sampled shrimp were individually weighed, and the hemolymph (approximately 200 μL) was extracted from the base of the first pereiopods (Figure 3.4.5.1) with an insulin syringe with a 28 gauge needle preloaded with 200 μL of cooled shrimp anticoagulant solution (450 mM NaCl, 10 mM KCl and 10 mM HEPES; pH 7.3). To obtain shrimp plasma, the mix (hemolymph:anti-coagulant; 1:1) was centrifuged 10 min. at 10,000 x g at 4 ºC, before storing plasma under –20 ºC (Mendoza, 1992).

3.4.5.2 Larvae samples (whole body sampling) for total RNA extraction

For the 1st and 2nd samplings, which represent the period before and after the nutritional stimulus, respectively (Figure 3.4.1). The whole body post-larvae sampled was washed on sterile water, removed the excess of water with an absorbent paper and immersed in RNA Later. Afterwards they were kept at 4ºC during 24 h and finally storage at –20ºC.

3.4.5.3 Tissue sampling for total RNA extraction

The 3rd (before challenge) and the 4th samplings (after challenge; Figure 3.4.1), juvenile shrimp were weighed and shrimp tissue was excised, i.e., hepatopancreas and caudal muscle without carapace, immediately immersed in RNA Later (Figure 3.4.5.3). Before the RNA extraction, tissues were filtered with a sterile mesh, removed the excess of buffer, weighed and immersed on Trizol solution.

3.4.6 Metabolites analyses

Plasma glucose, triglycerides and lactate were analyzed with Glucose RTU (BioMerieux, France), Triglycerides PAP 150 (bioMérieux, Craponne, France) and Lactate (Randox Laboratories, Crumlin, United Kingdom), respectively, through commercial kits adapted to microplates, adapted to microplates, according to recommendations of each manufacturer.
Figure 3.4.5.3. Hepatopancreas sampling. Source: Patricia Vieira.
3.4.7 DNA extraction

DNA isolation from the hepatopancreas was performed as previously described by Marandel et al. (2012). Briefly, 100 mg of hepatopancreas tissue (remaining samples of hepatopancreas after challenge phase from RNA extraction, CTL and RES; n=10), were digested overnight at 37°C under agitation in TNES buffer (125 mM NaCl, 10 mM EDTA, 17 mM SDS, 4 M Urea, 10 mM Tris–HCl, pH 8) containing 0.42 mg of proteinase K (Sigma Aldrich, P6556) at a ratio of 5.5 ml of TNES buffer per 100 mg of tissue. After adding one volume of phenol-chloroform-isoamyl alcohol (25:24:1) samples were mixed gently during 15 min and centrifuged for 15 min at 6000 g at room temperature. NaCl 5M (675 μL) was added to the supernatant. After mixing gently, 4 volumes of cold 100% ethanol were added and samples were centrifuged for 15 min at 8000 g at 4°C. DNA pellets were washed in 1 mL 75% ethanol and centrifuged again. Pellets were dried, re-suspended in water and treated with 2.4 μg RNase (Promega, A7973) for 1 h at 37°C. Finally, isolated DNA was quantified by NanoDrop (Thermo Fisher, USA) and the quality was verified on 1% agarose gel.

3.4.8 Global DNA methylation evaluation

DNA global methylation pattern (5-mC) was assessed using the MethylFlash Methylated DNA Quantification Kit (Epigentek, USA). Each analysis was performed in duplicate using 5.0 ng of DNA, following the manufacturer’s instructions.

3.5 Statistical analyzes

The results shown on this manuscript are presented as means ± standard deviation (SD). Statistical analyses were carried out using R software (v.3.1.0)/R Commander Package. The normality and the homogeneity of variance of the variables were tested with Shapiro-Wilk’s and Levene’s test, respectively. Different statistical tests were used according with the experiment and the type of data assessed by the non-parametric (Wilcoxon) test and parametric test (Student’s t-test, ANOVA and two-way ANOVA), followed by Tukey’s HSD test (P<0.05). For all analyses, P<0.05 was considered statistically different.
Chapter 4

Results
The data generated from the studies performed of this thesis, have been subjected of three peer-reviewed publications: among them the first was published, the second one has been submitted (under review), while the third one will be submitted soon. Therefore, in this section, the original scientific articles are presented chronologically, following the order of the objectives of the thesis.

**Publication 1**


**Publication 2**


**Publication 3**

Lage, L.P.A., Weissman, D., Serusier, M., Putrino, S.M., Baron, F., Guyonvarch, A., Tournat, M., Nunes, A.J.P., Panserat, S. The 4 days feeding restriction at the protozoea stage had a little effect at long term on metabolic gene expressions in whiteleg shrimp (*Litopenaeus vannamei*). *(To be submitted)*
Publication 1
4.1 I – Ontogenesis as a tool for the identification of critical windows to test the nutritional programming (1st article published in the international journal *Aquaculture*: + a poster at the Larvae Symposium, Ghent, Belgium, 2017)

**Summary of article**

**Objective**

The objective of the first paper was to define the developmental windows of the whiteleg shrimp *Litopenaues vannamei* to do later the environmental stimulus for testing the concept of nutritional programming. Thus, in the present study, we aimed to characterize the ontogenesis of the expressions of 20 genes coding for key proteins involved in nutrient use of the whiteleg shrimp. We did this work at 10 developmental stages from embryo up to post-larvae. The major molecular biomarkers involved in nutrient use, such as digestion; amino acids, lipid, glucose and energy metabolisms were investigated for the first time during the ontogenesis in shrimp.

**Experiment and analyses**

Sampling of shrimp larvae during ontogenesis were performed in a commercial hatchery during the cycle production. Shrimp were stocked as nauplii N3 on outdoor tanks (400 animals L\(^{-1}\)) and reared until post-larvae 10 (PL10) in a single static system. At protozoea stage, larvae were fed with a mix of microalgae (*Thalassiosira* spp and *Chaetoceros* spp) and a multiform formulated diet, whereas at mysis substage and post-larvae stages, *Artemia* were incorporated into the diet. The samples were performed at all stages and sub-stages of the embryo and larvae development. mRNA analyses were performed on the samples that compromise the different metamorphoses. The first sample took place at zygote phase, 8 h after the spawning (E 8h). Subsequent to post hatching, shrimp were collected during naupli sun-stage N5; zoea, sub-stages Z1, Z2, and Z3; mysis, sub-stages M1, M2, and M3, and; two post-larval sub-stages, PL1 and PL5. Trizol was used to extract the whole body RNA, then the gene expression of the target genes were analyzed through RT-qPCR. Statistical analyses, one-way (ANOVA), were carried out. Statistically significant differences were found at p<0.05, followed by the Tukey’s HSD test using R software (v.3.1.0)/R Commander Package.

**Main results**

The results of this study suggest the existence of two sensitive windows of the larvae development in whiteleg shrimp, characterized by high molecular plasticity for metabolism at the molecular level: the protozoea and early post-larvae stages.
In conclusion, our results shown the existence of two developmental windows of development of *L. vannamei*. The sub-stage zoea Z1 shows high molecular plasticity, which could be a promising stage for testing the nutritional programming. However, the fast development combined with the fragile animal characteristics of the protozoea, make this phase difficult for manipulation. Thus, we selected a second developmental window with high molecular plasticity also (the post-larvae stage) to be the preferential candidate for performing the early stimuli to modify metabolism at long-term.
Ontogenesis of metabolic gene expression in whiteleg shrimp (Litopenaeus vannamei): New molecular tools for programming in the future

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A B S T R A C T

Nutritional programming is a very promising strategy for modifying nutrient metabolism to better adapt animals to new diets. Although it is a well-known approach in fish-farmed species, it has never been applied in marine shrimp. A preliminary requirement to perform nutritional programming is to identify the best developmental window for early stimulus. The objective of the present study was to characterize the ontogenesis of the expression of the main genes involved in digestion and metabolism in white shrimp, Litopenaeus vannamei. Four genes involved in digestion, 11 genes in intermediary metabolism (glucose, amino acid and lipid metabolism) and 5 genes in energy metabolism (mitochondria) were studied at 10 different developmental stages (from eggs – 8 h after fertilization - up to post-larval substage 5) using qRT-PCR for measuring mRNA levels. Our data show that almost all of the studied genes present higher mRNA levels during the protozoa substage Z1, which could be explained by the endogenous-exogenous feeding transition in white shrimp. In conclusion, Z1 substage shows a high molecular plasticity for metabolism which becomes the preferential developmental window for future programming experiments in L. vannamei.

Statement of relevance: Our study aimed at characterizing for the first time the ontogenesis of expressions of genes involved in intermediary metabolism (nutrient metabolism). This approach determined developmental windows with high molecular plasticity (Z1 and PL stages). These data will support future studies in nutritional programming for the whiteleg shrimp by determining the timing for early stimulus.

1. Introduction

Prenatal or early nutritional neonatal events exerted at critical developmental windows may result in permanent changes in postnatal growth potential, health and metabolic status in mammals (Burdge and Lillycrop, 2010; Devaskar and Thamotharan, 2007; Duque-Guimarães and Ozanne, 2013; Lucas, 1998; Metges et al., 2014; Patel et al., 2009; Patel and Srinivasan, 2002). It has been suggested this process of developmental plasticity utilizes early nutritional cues to prepare individual phenotypes to better match the predicted future nutritional environment (Gluckman et al., 2005). Possible biological mechanisms for "imprinting" the nutritional event until adulthood in mammalian vertebrates, comprise adaptive changes on gene expression pattern or cellular phenotype (epigenetic phenomenon), nutrient-sensitive signaling pathways and adaptive clonal selection which may be transmitted to future offspring (Gut and Verdin, 2013; Lucas, 1998; Symonds et al., 2009; Waterland and Jirtle, 2003). Experimental data on the concept of nutritional programming in fish from recent studies have dealt with the possibility of altering the functioning of long-chain fatty acid desaturation in European seabass, Dicentrarchus labrax (Vagner et al., 2009, 2007), the use of dietary carbohydrates in rainbow trout, Oncorhynchus mykiss (Geurden et al., 2014; Geurden et al., 2007) or zebrafish, Danio rerio (Fang et al., 2014; Rocha et al., 2014) and, the acceptance and use of plant-based feed in rainbow trout (Geurden et al., 2013; Balasubramanian et al., 2016). Taken together, these results have shown particular long-term effects on molecular markers or on growth due to an acute early-life exposure to the imposed nutritional stimulus. This appears promising as a prospect for altering nutrient use in adult animal by nutritional programming at early stages.

The Pacific white shrimp (Litopenaeus vannamei) is the most...
important commercially farm-reared marine crustacean worldwide (FAO, 2016). In shrimp nutrition, the main objectives are to improve feed formulations through the development of alternatives diets with low levels of fish meal, incorporation of new plant proteins, decrease of dietary proteins and increase of carbohydrates (Gatlin et al., 2007; Guo et al., 2006; NRC, 2011; Rosas et al., 2001, 2000; Sá et al., 2013; Sabry-Neto et al., 2016; Sookying et al., 2011; Sookying and Davis, 2011). To improve the use of the alternative diets in juvenile shrimp, we hypothesized that the experiments of programming could be promising as shown recently in fish. However, as a crustacean, the species goes through a typical metamorphosis process during early development (embryo, nauplii, protozoa, mysis and, post-larvae steps) before it becomes an adult. Morphological and physiological features change dramatically over this developmental period (Dall et al., 1990) questioning the developmental window to perform the nutritional programming. Thus, investigations involving metabolic gene expression at early development and during metamorphosis process are mandatory before future experiments on nutritional programming can be conducted.

Thus, in this study, major molecular biomarkers involved in nutrient metabolism were investigated for the first time during the ontogenesis of shrimp. Some of these molecular markers were linked to digestion (Wei et al., 2014b), glucose metabolism (glucose transport, aerobic and anaerobic glycolysis, gluconeogenesis and blood sugar regulation (Chung et al., 2010; Cota-Ruiz et al., 2015; Fanjul-Moles, 2006; Lagomestri et al., 2007; Lee et al., 2014; Ma et al., 2009; Martinez-Quintana et al., 2014; Sánchez-Paz et al., 2008; Ventura-López et al., 2016)), amino acid metabolism (Lai et al., 2011; Li et al., 2011, 2009; Liu et al., 2012; Richard et al., 2010; Rosas et al., 2001), lipid metabolism (Yang et al., 2011) and mitochondria energetic metabolism (Jimenez-Gutierrez et al., 2013; Li et al., 2011; Martinez-Cruz et al., 2012; Verkhovsky et al., 2006) (Table 2). We characterized the ontogenesis of 20 mRNA levels of key proteins involved in nutrient use in whiteleg shrimp at 10 different developmental stages from embryo up to the post-larval stage 5 (PL5, i.e., 5 days after first metamorphoses into post-larvae). Some of these genes were previously described for the genes coding for digestive enzymes (lipase, preamy-lase, trypsin and chymotrypsin) (Wei et al., 2014b) and served, in the present study, as controls for the analysis of the other ones.

2. Material and methods

2.1. Shrimp rearing

Larvae of the Pacific whiteleg shrimp (Litopenaeus vannamei) were reared in a commercial hatchery, CELM Aquicultura, located 160 km distant from Fortaleza capital, NE Brazil. Shrimp were stocked as nauplii N3 (nauplius substage 3) at 400 animals/L and reared until post-larvae 10 (PL10) in a single-phase static outdoor tank system. Water quality management consisted of daily applications of a commercial probiotic, water exchange and temperature control. Daily water exchange at 10% of total tank volume only started when shrimp reached PL1. To maintain water temperature above 30 °C, tanks were kept under a greenhouse made of a translucent plastic cover. When stocking larvae, water temperature was raised and kept at 34 °C, but reduced progressively to ambient temperature as PL12 substage was reached. Dissolved oxygen, pH, temperature, and salinity were monitored daily. Total ammonia nitrogen, nitrite, nitrate and phosphate were monitored daily through colorimetry. At zoea stage larvae were fed with a mix of microalgae (Thalassiosira spp. and Chaetoceros spp.) and multiform formulated diet, while at mysis substage and post-larvae Artemia was inoculated into the diet herewith a multiform diet.

2.2. Shrimp sampling

Shrimp larvae were sampled at 10 different developmental stages starting at the time immediately after shrimp spawning until PL5 stage (Table 1). These samples comprised the main metamorphoses over the whole larval development of the white shrimp. The first sampling took place at zygote phase, 8 h after spawning (E 8 h). Subsequent to post hatching, shrimp were collected during nauplii, sub-stage N5; zoea, sub-stages Z1, Z2, and Z3; mysis, sub-stages M1, M2, and M3; and two post-larval sub-stages, PL1 and PL5 (Wei et al., 2014a, 2014b). For each developmental stage, five pools containing 10 individuals (whole body) were collected.

For sampling, shrimp larvae were washed with sterile salt water, and dried with an absorbent paper. Samples were preserved on a RNA Stabilization Solution (RNA Later Sigma®). 100 mg tissue/1 mL RNALater, immediately immersed on this solution which was kept frozen at −20 °C. Before sampling, visual evaluations under a microscope were performed to ensure at least 90% of the individuals at sampling corresponded to the desired developmental stage (Trece and Fox, 1999; Wikins and Lee, 2002; Juarez and Moss, 2010; Wei et al., 2014a).

2.3. mRNA levels analysis

Biological material was collected with a sterile mesh (100 μm) and then dried with an absorbent paper to remove the maximum amount possible of RNALater. Subsequently, the tissue was weighed and immediately immersed on a Trizol solution. RNA extraction, from whole body, was performed using the reagent Trizol® (100 mg sample/1 mL Trizol). The concentration was determined through the
One-Way (ANOVA) analyses of variance were carried out to examine individual differences (R software, R Commander Package).
3. Results

3.1. Ontogenesis of the expression of four enzymes involved in the digestion process: molecular validation of the study

The ontogenic expression of the four enzymes involved in carbohydrate digestion (preamylase), lipid digestion (triacylglycerol lipase) and protein digestion (trypsin and chymotrypsin) is presented in Fig. 1. First, there was a significant increase in preamylase gene expression at Z1 substages, followed by a significant decrease at Z2, then a new increase at Z3 and a decrease at M1 substages (Tukey’s HSD, p < 0.05). The profile of expression of triacylglycerol lipase and trypsin are similar to the preamylase one: there is a significant peak of gene expression during the three protozoea substages, Z1, Z2 and Z3 (Tukey’s HSD, p < 0.05). By contrast, chymotrypsin was higher and expressed only at final stages of post-larvae, substage PL5 (Tukey’s HSD, p < 0.05).

3.2. Ontogenesis of the gene expressions for protein involved in amino acid and lipid metabolisms

The ontogenic expression of the genes coding for two enzymes involved in amino metabolism glutamate dehydrogenase - GDH and glutamine synthase - GS is shown in Fig. 2. GS gene expression increase significantly at Z1 substage (Tukey’s HSD, p < 0.05) and then decrease at protozoea Z2 and Z3 before returning to the basal level at the mysis and PL substages (Tukey’s HSD, p < 0.05). By contrast, GDH gene expression increases at the nauplii stage, with a peak at protozoea Z1 (Tukey’s HSD, p < 0.05) before returning to a level similar observed at the nauplii stage.

3.3. Ontogenesis of the gene expression for proteins involved in energy metabolic process (mitochondria)

The ontogenic expression of the nuclear genes coding for proteins involved in phosphorylation oxidative pathway in mitochondria (i.e., the cytochrome C oxidase genes subunit VI (a, b and c) and the two ATP synthase (a and b) genes) is shown in Fig. 3. The same pattern of gene expression was detected for all the five genes: a peak of gene expression at protozoea Z1 followed by a progressive decrease of mRNA levels (Tukey’s HSD, p < 0.05). At mysis, mRNA levels are similar to the first stages, embryo - 8 h and nauplii N5.

3.4. Ontogenesis of the gene expressions for proteins involved in glucose transport and metabolism and the hormone CHH

The ontogenic expression of the genes coding for two glucose transporters (Glut1 and Glut2) and some key enzymes involved in glycolysis (HK, PK), gluconeogenesis (FBPase, PEPCK) and lactate metabolism (LDH) is shown in Fig. 3. Profiles of gene expression are more diverse here compared to data described previously. Indeed, a peak of gene expressions at protozoea Z1 substage is also observed for the two glucose transporters and the glycolytic enzymes (HK and PK). This peak is followed by a decrease of gene expression up to the PL stages for HK and Glut2. However, this was not the case for Glut1 and PK which were expressed at high level at the post-larval stages (PL1 and PL5) (Tukey’s HSD, p < 0.05). LDH gene presents a progressive increase of expression from the protozoea Z1 up to post-larval PL5 substage. In regards to gluconeogenesis, FBPase gene expression increases at the nauplii stage (Tukey’s HSD, p < 0.05) and slightly decreases to a plateau. PEPCK gene expression is atypical with an increase of expression only at the PL substages. Finally, the gene coding for the CHH hormone (linked to glucose homeostasis and metabolism) is also higher expressed at protozoea Z1 substage and, after a decrease, it rises again at PL stage.

4. Discussion

This study aimed at analyzing the ontogenesis of several targeted genes involved in nutrient use (transport and metabolism) in whiteleg shrimp. From our understanding, it is the first time this was carried out with L. vannamei. The study also investigated the molecular profile for these genes in order to provide new tools for future development of nutritional programming for this species. The profiles of expressions for the digestive enzymes (preamylase, triacylglycerol lipase, trypsin and chymotrypsin) are similar to what has been previously shown (Wei et al., 2014b). These data validate our experiments for the analysis of the ontogenesis for all the other metabolic genes.

4.1. Zoe stage: a stage of high molecular plasticity for metabolism

Most of the studied genes (except chymotrypsin gene), a strong peak of gene expression (3-5 fold increase) was observed in shrimp at the protozoea Z1 compared to the nauplii N5 substage. Clearly, at a

For many metabolic genes (except LDH and FBPase genes), we observed after an increase at the protozoea Z1 stage of mRNA levels for the metabolic genes, a significant decrease of gene expressions in the following stage (protozoea Z2 and Z3 and mysis). At the protozoea stage, white shrimp are classified as a phytoplankton feeder followed by a zooplankton consumer at the mysis stage. Phytoplankton is rich in proteins like the zooplankton, but the latter is poor in carbohydrates (Fyhn, 1989; Juarez and Moss, 2010; Le Vay et al., 2001; Puello-Cruz et al., 2002); this variation in dietary protein/carbohydrates can explain the maintenance of trypsin gene expression and a decrease of amylase gene expression in relation to digestion (Le Vay et al., 2001; Wei et al., 2014b). Thus, the changes in diet quality during the protozoea-mysis stages along with the modification of allometry parameters for some organs, such as liver/muscle (we analyzed here the whole body mRNAs), could also explain also the global decrease of gene expression for metabolic actors. However, future studies are needed in order to better understand the mRNA levels after the
Fig. 3. Ontogenesis of gene expression for proteins involved in glucose transport and metabolism (glycolysis and gluconeogenesis). Analysis has been performed using real time PCR. The mRNA levels measured at each developmental stage are shown as means (5 pools) with standard deviation. E-8 h: eggs after fertilization (8 h). N5: nauplii stage 5. Z1: protozoea stage 1. Z2: protozoea stage 2. Z3: protozoea stage 3. M1: mysis stage 1. M2: mysis stage 2. M3: mysis stage 3. PL1: post-larvae stage 1. PL5: post-larvae stage 5. Different letters indicate statistically significant differences of expression between stages of development (Tukey’s HSD, p < 0.05).

protozoea stage.

4.2. Post-larval stage: a second peak of mRNA levels for metabolic genes

For some specific genes involved in glucose metabolism and transport, we observed a second peak of gene expression at the post-larval stages (PL). Indeed, PK, LDH, Glut1 and CHH are highly expressed at the PL stages. We have no clear hypothesis to explain this second peak of expression. However, it suggests that at the PL stages the use of glucose as an energy source (aerobic and anaerobic glycolysis) (Enes et al., 2009; Gut and Verdin, 2013; Hemre et al., 2002; Olson and Pessin, 1996; Pilkis and Granner, 1992) in all the tissues (glut1 an ubiquitous transporter of glucose (Jones and Bergeron, 2001; Martinez-Quintana et al., 2014; Menoyo et al., 2006; Olson and Pessin, 1996; Wang et al., 2016)) could be more important than in the previous developmental stages (in relation also to the CHH gene expression). Finally, although this peak is not fully understood, the PL stage could be also a potential candidate for programming stimulus. The main advantage compared to the protozoea 1 stage is that the PL stage is longer and that the animals are less fragile and eat inert diets. However, the main inconvenient is that this stage could be a little too late for programming some metabolic targets at a molecular level.

In conclusion, our study suggests strongly that the protozoea Z1 substage in whiteleg shrimp is a developmental window showing a very high metabolic plasticity at least at the molecular level. This stage is probably a promising stage for testing different environmental stimuli (especially nutritional factors) to modify at long-term metabolism in shrimp for a better use of nutrients associated with improved growth performance. Since protozoea Z1 is not easy to manipulate (short-time stage associated with potential fragile animals), a secondary develop-
mental window that could act as a potential candidate for a stimulus and programming is the PL stage.

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References


Publication 2
4.2 II – Nutritional programming of shrimp at the post-larvae level (2nd article; submitted in the international journal Aquaculture + a poster at the ISFNF, Las Palmas, 2018)

Summary of the article

Objective

In the present study, we aimed to assess for the first time the nutritional strategy based on the nutritional programming concept in order to modify at long term the nutrient use in whiteleg shrimp *L. vannamei*. Based on our previous study, a strong feed restriction stimulus was applied in post-larvae 1 (PL1) during three days, i.e. a 70% feed restriction (70% less feed than normal feed allowance - RES) compared with a normal feeding protocol BernAqua (CTL). Then, all the groups after a common rearing were challenged for 70 days with 3 diets containing different protein/carbohydrate ratios.

Experiment and analyses

Animals at the post-larvae 1 stage (PL1; 1.10 ± 0.10 mg body weight) purchased on a commercial hatchery, were transferred to the laboratory, stocked on 3 m³ circular tanks under 1.6 PL L⁻¹, and subjected to a feed restriction stimulus. Afterwards, animals were raised on the same conditions up to 0.9 ± 0.10 g, and at the 50th day animals were transferred to 1.5 m³ rearing tanks to the challenge phase with the experimental diets.

Diets were produced on small scale extruder. First of all, a basal diet was formulated to contain 15.0% fishmeal (% of the diet, as is basis) 43.3% crude protein (CP, dry matter basis, DM) and a NFE and CP (nitrogen-free extract) ratio of 0.7. Afterwards, two others diets were designed by replacing fishmeal with wheat flour and soybean meal, so that CP was reduced to 36.9 and 29.6% (diets P37, and P30, respectively) with NFE/CP ratios of 1.0 and 1.5, respectively.

The effect of stimulus and challenge were evaluated through analysis of growth performance, measures of plasma metabolites and analysis of metabolic biomarkers at the molecular level in muscle and hepatopancreas at different periods (after the early stimulus, before the dietary challenge and after the dietary challenge).

Main results

The direct effects of the feed restriction on the intermediary metabolism/digestion of PL 4, was evaluated through analysis of the mRNA levels. No statistical difference on the mRNA levels was observed for many of the genes, except for the *trypsin* gene; this gene was down-regulated in the feed restricted larvae. This result can be related to the fact that protein, the main component
of the diet, was reduced. No deleterious effect was observed on the shrimp growth performance, evaluated after 12 rearing days (hatchery phase), allowed to conclude that no difference in survival between the two experimental groups.

The final body weight and specific growth rate (SGR) were higher in the feed restricted group (linked to a programming effect). The high protein diet also had a positive effect on the final body weight and FCR as expected, which are linked with to the differences in the protein intake.

Regarding the gene expression on the intermediary metabolism after the challenge phase, a significant down-regulation for the genes coding for digestive enzymes (*lipase, preamylase, and trypsin*), energy metabolism (*cox VI b*), amino acid metabolism (*gs*) and glucose metabolism (*lvglut 1, lvglut 2 and pk*) was associated with the early nutritional (feed restriction) history. Whereas the effects of the diets suggest that shrimp adapt well to the decrease of the proteins/carbohydrate ratios by decreasing some mRNA levels coding for amino acid catabolism and gluconeogenesis.

In conclusion, our study demonstrates for the first time the possibility to program shrimp at long term due to an early nutritional stimulus, as it was observed in mammals (nutritional programming). Indeed, modification of growth performance as well as the modifications of some gene expressions involved in metabolism were associated with the early feed restriction.
Metabolic programming in juveniles of the whiteleg shrimp (Litopenaeus vannamei) linked to an early feed restriction at the post-larval stage

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ABSTRACT

In this study, the concept of metabolic programming has been tested for the first time in whiteleg shrimp (L. vannamei). Shrimp were raised under a 70% feed restriction during the post-larval stage over three days and compared to a control group. After 46 days, shrimp were challenged with 3 diets showing different nitrogen free-extract: crude protein ratios (1.5, 1.0 and 0.7) for 70 days. In order to test the existence of metabolic programming, we analyzed shrimp growth performance as well as mRNA levels of different metabolic and digestive actors after the stimulus, and also before and after the challenge. No direct effects of the stimulus were observed for several digestive and metabolic actors, except for the trypsin mRNA (lower in the feed-restricted group, probably linked to a decrease in dietary protein intake). As expected, significant effects associated with the diet challenge were detected for shrimp performance, i.e., growth was lower in shrimp fed with reduced levels of dietary proteins. More interestingly, some effects linked to the nutritional history were also detected showing an improved growth performance for shrimp previously restricted at the post-larvae stage. After the dietary challenge, significantly lower mRNA levels for hepatopancreatic genes involved in digestion (lipase, preamylase and trypsin), amino acid metabolism (gs), energy metabolism (cox VI b) and glucose metabolism (logl bt 1, logl bt 2 and pk) were found in restricted shrimp. The link between an enhanced growth performance and these molecular markers in early feed restricted shrimp requires further studies. Overall, our study has demonstrated for the first time that shrimp can be programmed by an early nutritional stimulus. This will allow the development of new feeding strategies in shrimp for sustainable aquaculture.

1. Introduction

Marine shrimp accounts for 22.6% of the total aquaculture value (US$ 160.2 billion) and < 10% of the global aquaculture production (FAO, 2016). Despite the successful expansion of farmed shrimp production worldwide, there is an increasing need to adopt feeds free from wild-caught fish to ensure its future development and sustainability. Fishmeal (FM) is the preferable protein source in industrial feed mills due to its high digestibility, nutritional value and palatability (Suárez et al., 2009; Tacon and Metian, 2008). Much of the FM used in aquafeeds still originates from capture fisheries of small pelagic fish species, e.g., anchoveta, sardines, mackerel. Fish landings have now reached their maximum capacity, thus supplies are limited (FAO, 2016; Naylor et al., 2009). FM also represents the most expensive protein source in a shrimp feed formulation (Naylor et al., 2009; Tacon and Metian, 2008; Williams, 2007).

In shrimp feeds, FM can be replaced by alternative protein sources, byproducts obtained from agriculture and from the rendering animal

Abbreviations: atpase a, Litopenaeus vannamei mitochondrial atp synthase subunit alpha precursor, mRNA; atpase b, Litopenaeus vannamei mitochondrial atp synthase subunit beta precursor, mRNA; chymotrypsin, Litopenaeus vannamei chymotrypsin b II mRNA; eos V I a, Litopenaeus vannamei mitochondrial cytochrome C oxidase subunit VIIa precursor, mRNA; eos V I b, Litopenaeus vannamei mitochondrial cytochrome C oxidase subunit VIIb precursor, mRNA; eos VI c, Litopenaeus vannamei mitochondrial cytochrome C oxidase subunit VIIC precursor, mRNA; ef1a, Litopenaeus vannamei elongation factor 1-alpha mRNA; fis, Litopenaeus vannamei fission factor mRNA; fisf, Litopenaeus vannamei fructose 1,6-bisphosphatase mRNA; gs, Litopenaeus vannamei glutamine synthetase mRNA; gdh, Litopenaeus vannamei glutamate dehydrogenase mRNA; idh1, Litopenaeus vannamei isocitrate dehydrogenase mRNA; idh2, Litopenaeus vannamei lactate dehydrogenase mRNA; lipase, Litopenaeus vannamei lipase mRNA; luciferase, Litopenaeus vannamei luciferase mRNA; pepk, Litopenaeus vannamei phosphoenolpyruvate carboxykinase gene; pk, Litopenaeus vannamei pyruvate kinase mRNA; promyelase, Litopenaeus vannamei preamylase 1 (Litopenaeus vannamei); trypsin, trypsin (Litopenaeus vannamei)

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industry. However, dietary replacement of FM can only be carried out with adequate supplementation of essential nutrients, such as amino acids, fatty acids and minerals (Nunes et al., 2011, 2014; Tacon and Metian, 2008). Alternative proteins may contain from 40 to 70% crude protein, but they are often associated with a low nutrient digestibility, unbalanced amino acid profile, anti-nutritional factors, poor attractability and a high carbohydrate content which result in negative effects on growth performance and feed efficiency (Francis et al., 2001; Gatlin et al., 2007; Nunes et al., 2006a). Carbohydrates (CBH) are the cheapest nutrients in aquatic feeds, acting as one of the main energy sources. When digestible CBH are used at 20 to 30% in the diets, they can spare energy sources from proteins and lipids. However, when CBH are ingested at high levels, they can lead to poor growth performance and survival (Cuzon et al., 2004; Gatlin et al., 2007; Guo et al., 2006; NRC, 2011; Pascual et al., 2004; Shiau, 1998; Wang et al., 2016). Thus, the development of shrimp aquafeeds containing lower levels of protein (especially derived from FM) and higher levels of carbohydrates can be beneficial for the shrimp nutrition.

The concept of nutritional programming has been largely studied in mammals i.e. environmental factors (nutritional, pH, oxygen, etc.) exerted during the early development may have long-term effects in the physiology and metabolism functions during adulthood (Burdge and Lillycrop, 2010; Devaskar and Thamotharan, 2007; Duque-Guimarães and Ozanne, 2013; Lucas, 1998; Metges et al., 2014; Patel and Srinivasan, 2002). This concept was recently tested in fish but shown promising results on species including the rainbow trout, Oncorhynchus mykiss (Balasubramanian et al., 2016; Geurden et al., 2007, 2013, 2014; Liu et al., 2017; Panserat et al., 2017), European seabass, Dicentrarchus labrax (Vagner et al., 2007, 2009), Atlantic salmon, Salmo salar L. (Clarkson et al., 2017; Vera et al., 2017) and in zebrafish, Danio rerio (Fang et al., 2014; Rocha et al., 2014). For examples, in rainbow trout, Geurden et al. (2014) reported that an early nutritional stimulus at first-feeding stage (i.e., hyperglucidic diets) induced persistent modifications in the mRNA levels of the genes related with the glucose metabolism, whereas a metabolic long-term effects on the energy metabolism related with the vitamin intake at first-feeding has been recently shown (Panserat et al., 2017). However, to our knowledge, no study on metabolic programming has been performed on crustaceans.

Our objective in the present study was to use the strategy of metabolic programming in order to modify the use of nutrients in whiteleg shrimp. The best developmental window for the early stimuli was determined previously; indeed, Lage et al. (2017), through mapping key metabolic biomarkers over the early larval development in whiteleg shrimp, suggested that two developmental stages, i.e., protozoa sub-stage 1 Z1 and post-larvae stage PL1, could be the preferential periods to test early stimuli. Here, we hypothesized that a caloric restriction stimulus (a strong environmental driver for the programming in mammals (Barker and Osmond, 1986; Lucas, 1998; Spencer, 2012)) during the post-larvae stage (i.e., PL1 phase) could improve the use of dietary nutrients in adults. Precisely, the purpose of this study was to evaluate the long-term effects of an early stimulus, consisting in a feed restriction (70%) applied at the PL stage, on juvenile shrimp challenged with 3 diets (different protein/carbohydrate ratios) during 70 days.

2. Material and methods

2.1. Water preparation and post-larvae stocking

Seawater was pumped into two 20 m$^3$ reservoirs located at LABOMAR’s experimental aquaculture facility (Eusébio, Brazil). By this time, water salinity was adjusted to match at the hatchery salinity (28 ups) by adding fresh water disinfected with chlorine (0.02 g L$^{-1}$). Subsequently, water was sand-filtered for 24h to remove large particles, and then filtered by a carbon filter. This water was used to fill six circular fiberglass tanks of 3 m$^3$. Water of each tank was fertilized with 100 g of a grinded commercial starter shrimp feed and 200 g of dried sugar-cane molasses. Strong aeration was supplied through mechanical blowers to provide sufficient dissolved oxygen (DO) and water circulation for plankton growth.

Two days after water fertilization, 30,000 post-larvae (PL) of L. vannamei at post-larval stage 1 (PL1) were brought to the lab from commercial hatchery (Potiporã Aquaculturra Ltda., Beberibe, Brazil) in plastic bags containing seawater saturated with DO. At arrival, PLs were stocked equally in each rearing tank under 1.6 PL L$^{-1}$. During stocking, water quality was carefully monitored to slowly adjust PLs. During PL
rearing, tanks were shaded to avoid excessive growth of phytoplankton which could interfere with the nutritional stimulus.

2.2. Shrimp rearing and feeding

Immediately after transportation, PLs were acclimated and stocked in rearing tanks (Fig. 1). This was followed by a three-day feeding stimulus (three days), hatchery and nursery phases which lasted 47 days. Subsequently, juvenile shrimp were transferred to 30 rearing tanks of 1.5 m³ to perform a long-term dietary challenge (70 days). Water salinity, pH and temperature during the hatchery culture and diet challenge stages fell within acceptable ranges for the culture of L. vannamei (29 ± 1.04 g L⁻¹, 8.04 ± 0.10; 28.8 ± 0.75 °C; 40 ± 1.20 g L⁻¹, 7.9 ± 0.19; and, 30.9 ± 1.13 °C), respectively.

2.3. Stimulus protocol

For the early nutritional stimulus, PLs of 1.10 ± 0.10 mg body weight were divided into one experimental group and a control (CTL) group with three replicate tanks each. Post-larval shrimp were fed for three days, following a commercial hatchery feeding guide (BernAqua NV, Olen, Belgium). The CTL group were fed under a regular feed allowance while the feed-restricted treatment (RES) were subjected to a 70% feed reduction (Table 1). During the stimulus period, no live feed (Artemia spp. and microalgae) was delivered to PLs although it could be naturally available in water. Thus, feed restriction was based solely on limiting allowance of inert food. Finished the stimulus, the shrimp were acclimated for one week with a commercial grower shrimp feed (Density 38), Vitellus Standard (BernAqua NV, São Lourenço da Mata, Brazil). The rearing system was composed of 30 round tanks of 1.5 m³ (bottom surface area of 1.60 m²). Tanks operated under green-water conditions and a weekly water exchange of 10% of total water volume. Water was aerated continuously to reach near dissolved oxygen saturation (above 5 mg L⁻¹). Water pH, temperature and salinity were monitored once daily in the afternoon in each tank. Once a week, shrimp were sampled and weighed to adjust feeding.

2.4. Transition (common) rearing period

It is important to note that after the stimulus, shrimp were reared on the similarly until the beginning of the challenge phase. Indeed, shrimp derived from the CTL and RES groups were reared under the same conditions during 47 days before the dietary challenge study. At the end of this “common” period, shrimp have been challenged with 3 different diets during 70 days, from day 57 up to day 127 (Fig. 1).

2.5. Dietary challenge experimental design

Animals weighing 0.9 ± 0.10 g (mean ± standard deviation; n = 1680, CV = 11%) were reared in outdoor tanks with 30 shrimp m⁻² for 70 days (56 shrimp tank⁻¹). Prior to the dietary challenge, shrimp were acclimated for one week with a commercial grower shrimp feed (Density 38), Neovia Nutrição e Saúde Animal Ltda., São Lourenço da Mata, Brazil). The rearing system was composed of 30 round tanks of 1.5 m³ (bottom surface area of 1.60 m²). Tanks operated under green-water conditions and a weekly water exchange of 10% of total water volume. Water was aerated continuously to reach near dissolved oxygen saturation (above 5 mg L⁻¹). Water pH, temperature and salinity were monitored once daily in the afternoon in each tank. Once a week, shrimp were sampled and weighed to adjust feeding.

2.6. Feed formulation for the dietary challenge

Both CTL and RES groups were challenged for a 70-day period with three different diets in 30 tanks (5 dietary treatments × 2 groups × 5 replicates). First, a basal diet (P43) was formulated to contain 15.0% fishmeal (% of the diet, as is basis), 43.3% crude protein (CP, dry matter basis, DM) and a NFE (nitrogen-free extract)/CP ratio of 0.7. Afterwards, two others diets were designed by replacing fishmeal with wheat flour and soybean meal, so that CP was reduced to 36.9 and 29.6% (diets P37 and P30, respectively) with NFE/CP ratios of 1.0 and 1.5, respectively (Table 1). Diets were manufactured with a laboratory extruder using a 1.8 mm die. Proximate composition was carried out in each feed using standard methods (AOAC, 2002). NFE/CP ratios and dietary protein were 1.5, 1.0 and 0.7 for P30, P37 and P43, respectively. Shrimp were fed four times a day at 0700 am, 1000 am, 1300 pm and 1600 pm, exclusively in feeding trays. Daily meals were adjusted weekly based on shrimp weight gain. At the harvest, shrimp were weighed to a 0.01 g electronic scale and counted to determine their final body weight (FBW, g), specific growth rate (percentage increase in size per day – SGR; % day⁻¹, as is based SGR = [(lnFBW – lnIBW)/Δt (days)] × 100), final survival (%), gained yield (g of biomass gained m⁻³). Feed conversion ratio (FCR = AFI/Final gained biomass) was determined based on apparent feed intake. (AFI, g of total feed delivered per stocked shrimp). Daily, before each meal, all uneaten feed was collected and stocked under ~20 °C. At the end of the experimental period, feed remains were oven-dried and weighed to calculate AFI (Façanha et al., 2016; Nunes et al., 2006).

2.7. Shrimp sampling

All sampled shrimps were preserved on a RNA Stabilization Solution (RNA Later, Sigma®), with 100 mg tissue immediately immersed in 1 mL RNA Later, and then kept frozen at −20 °C. For the PL shrimp during the 1st (before stimulus) and 2nd sampling (after stimulus; Fig. 1), shrimp whole body was sampled, washed with sterile salt water, dried with an absorbent paper and immersed in RNA Later. For the 3rd (before challenge) and the 4th samplings (after challenge; Fig. 1), shrimp tissue was excised, refrigerated, and immersed in RNA Later and kept under −20 °C. Hepatopancreas and caudal muscle without carapace, immediately immersed in RNA Later, and kept under −20 °C. Hepatopancreas is the main tissue on crustaceans related to the nutrient digestion, absorption and metabolism, whereas muscle is related to the use of nutrients as energy sources (mainly for growth).

Table 1
Feeding protocol (g of diet per day) used for stimulus of post-larval L. vannamei (between PL1 and PL4 stage). CTL: control group. RES: feed restricted group (70% restriction).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>Feeding Protocol (g, day⁻¹, as is)¹</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Diet 1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.037</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.015</td>
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<tr>
<td></td>
<td>2</td>
<td>0.511</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.505</td>
</tr>
<tr>
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<td>0.96</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.037</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.015</td>
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</table>

Proximate composition (%, as is)²

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<th></th>
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<th>RES</th>
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</thead>
<tbody>
<tr>
<td>Crude protein</td>
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<td>54.0</td>
</tr>
<tr>
<td>Lipids</td>
<td>10.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Ash</td>
<td>10.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Fiber</td>
<td>3.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Nitrogen free extract</td>
<td>17.5</td>
<td>16.0</td>
</tr>
<tr>
<td>Moisture</td>
<td>8.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Ratios of nutrients/ crude protein

| Nitrogen free extract | 0.34 |
| Crude protein         | 0.24 |

¹ Feeding guide based on BernAqua NV (Olen, Belgium) recommendations.
² Vitellus Standard (BernAqua NV, Olen, Belgium). Particle size from 125 to 400 μm.
³ Royal Caviar (BernAqua NV, Olen, Belgium). Microcapsulated larval diet. Particle size from 100 to 200 μm.
⁴ Royal Seafood (BernAqua NV, Olen, Belgium). Special diet for post-larval stages. Particle sizes from 100 to 200 μm.
⁵ Product label. Manufacturer guarantee nutrient levels.
⁶ NFE, calculated by difference (100 – crude protein – crude fiber – crude fat – ash).

339
For the 4th sampling, shrimp hemolymph was collected (hemolymphanti-coagulant; 1:1). All samplings were done 3 h after the last meal based on a study performed with Penaeus monodon (Richard, 2010; data unpublished) which is equivalent to the species postprandial peak. Indeed, the author evaluated the postprandial glucose plasma kinetics of the black tiger shrimp from 0 h up to 6 h after the last meal (diets with different proteins/ carbohydrates ratios) and observed that the peak of the plasmatic glucose level was at 3 h after the last meal. To obtain the plasma, the mix (hemolymphanti-coagulant; 450 mM NaCl, 10 mM KCl and 10 mM HEPES; pH 7.3) was centrifuged 10 min. at 10,000 g at 4 °C, before storing plasma under −20 °C (Mendoza, 1992).

2.8. Plasma metabolites analysis

Plasma glucose, triglycerides and lactate were analyzed with Glucose RTU (BioMérieux, France), Triglycerides PAP 150 (bioMérieux, Craponne, France) and Lactate (Randox Laboratories, Crumlin, United Kingdom) kits, respectively, according to recommendations of each manufacturer.

2.9. Total RNA extractions and relative quantification of mRNA levels

Biological material was collected with a sterile mesh (100 μm) and then dried with an absorbent paper to remove the maximum amount possible of the RNA Later product. Subsequently, the tissue was weighed and immediately immersed in a Trizol solution. RNA extractions, from whole body (1st and 2nd samplings), hepatopancreas (3rd and 4th samplings) and muscle (3rd and 4th samplings), were performed using the reagent Trizol® (100 mg sample/1 mL Trizol), following manufacturer recommendations. The RNA concentrations were determined using the spectrophotometer NanoDrop 2000, whereas the RNA qualities were determined after migration on a 1% agarose gel electrophoresis.

Gene expression levels were determined using the real-time RT-PCR (n = 6 RNA samples per treatment). The mRNA levels of 19 genes coding for proteins involved in macronutrient digestion, nutritional metabolism (amino acid, lipid and glucose), glucose transport and mitochondrial metabolism were quantified using shrimp specific primers. The primer sequences used in the real-time RT-PCR assays are the same as those were recently reported by Lage et al. (2017).

For the RT-PCR, an amount of 1 μg RNA was reverse transcribed to cDNA with SuperScript III RNase H-Reverse Transcriptase Kit (Invitrogen) with random primers (Promega, USA). Real-time PCR was performed in the LightCycler 480 (ROCHE, Hercules, CA, USA). Quantitative PCR (Q-PCR) analyses for gene expression were performed using a reaction mix of 6 μL per sample containing 2 μL of the RT product (diluted cDNA), 0.24 μL of each primer (10 μmol L−1), 3 μL Light Cycler 480 SYBR® Green and 0.54 μL DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany) as previously described (Dai et al., 2014). Melting curves were systematically monitored (temperature gradient at 0.5 °C/10 s from 55 to 94 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each q-PCR run included duplicates of samples (reverse transcription) and negative controls (wells without reverse transcriptase, mRNA and cDNA). Relative quantification of target gene expression was performed using the ΔCT method (Pfaff, 2001). The relative quantification of mRNA levels of target genes was normalized with L. vannamei elongation factor 1-alpha (eflα) gene for whole body PL4 post stimulus analysis, for hepatopancreas analysis (at Day 50th and post challenge) and luciferase gene (exogenous Promega®) for muscle post challenge analysis. In all cases, PCR efficiency (E) was measured by the slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency values ranged between 1.8 and 2.2.

2.10. Statistical Analyses

Statistical analyses were carried out using R software (v.3.1.0)/R Commander Package, for the shrimp growth performance, molecular and biochemical analyses. Prior to statistical analyses, assumption of data normality and homogeneity of variances were assessed using the Shapiro-Wilk and Levene’s tests, respectively.

Before challenge, the growth performance (final body weight and PL g−1, number of PL per gram) and final survival (%) were evaluated by using Student’s t-test. Data are presented as mean ± SD (standard deviation, n = 3; experimental tank per experimental group), and were considered significantly different when P < .05. For the gene expression analysis (n = 6 samples per experimental group) after stimulus and before challenge, statistical differences were evaluated by Wilcoxon test as data did not follow a normal distribution.

After challenge, two-way analysis of variance (ANOVA) arranged in a 2 × 3 factorial design was used to determine the relation between stimulus (early nutritional history) and dietary challenge and their interactions. When stimuli × diet interactions were significant, means of all treatments were compared by Tukey’s HSD test (P < .05) using data obtained in the growth performance (n = 5, mean of each experimental condition), plasma metabolite (plasma metabolites, n = 9 samples per group) and mRNA studies (n = 6 samples per group).

3. Results

3.1. Early nutritional stimulus: growth performance and mRNA levels for metabolic genes

Shrimp performances were analyzed after the early stimulus, corresponding to the end of the hatchery phase (Table 3). No significant differences between shrimp from the control and the feed-restricted groups were observed on survival, final body weight and number of post-larva PL4−1 or PL4−1.

In order to confirm that the developmental stage of PL shrimp (between PL1 and PL4; analyzed in the control group) was a window of high molecular plasticity, the two stages PL1 and PL4 were compared at a molecular level. Overall, higher levels of mRNAs for several genes were observed at the PL4 stage compared to the PL1 stage (P < .05; Fig. 2). In regards to genes involved with digestion, only chymotrypsin gene expression did not differ between the two developmental stages; all the others were expressed at a higher level at PL4 stage. On the other parts, for intermediary metabolic genes, all the mRNA levels were higher in PL4 stage (P < .05) except for the ldh1 gene which was down regulated (2 fold-decreased) when larvae were feed restricted. Also, because feed restriction stimulus did not affect strongly genes related to digestion and intermediary metabolism, some other biomarkers associated with feed restriction process in mammals, such as those related to autophagy (bip, p62) and oxidative stress (lival, lametalo and licoa4c1), were also evaluated but no significant difference were also detected (data not shown).

3.2. Dietary challenge experiment: growth performance, plasma metabolites and mRNA levels for metabolic genes

We analyzed shrimp after the dietary challenge (Fig. 1) for several parameters. Shrimp were individually weighed at stocking, i.e., day 50
Table 5. Firstly, the e

Table 7. Observed when shrimp were fed with the P43 diet (higher protein in-

Table 8. Results of the molecular effects linked to the stimulus history?

Fig. 2. mRNA levels of the digestive and metabolic genes before (day 1) and after the stimulus (day 4) for the animals fed on the normal feeding protocol (CTL). Genes involved in glucose transport (lglut1 and lglut2), glycolysis (kh, Pk), gluconeogenesis (pepck, fbp), amino acid metabolism (gdh, gs), lipid metabolism (fas), energy metabolism (cox, atpase) and digestion (preamylase, trypsin, chymotrypsin, lipase) were analyzed. mRNA levels analyses were performed on total RNA extracted from whole body. Data represent means ± SD (n = 6 individual per groups). Statistical differences in mRNA levels (normalized by the exogenous Luciferase gene) between samples were evaluated in group means by Wilcoxon test; data were not normally distributed assessed by Shapiro-Wilk test, using statistical test (R-software). * means that the target gene is differentially expressed between the day 1 group and day 4 group (p < .05).

(n = 1680 shrimps) and at harvest, i.e., day 127 (n = 1630 shrimps) to determine their growth performance (Table 5). Statistical differences were observed in shrimp performance, except for the final survival and AFI parameter (P > .05). Statistically significant effects of the nutritional history (i.e., a programming effect) were detected for the final body weight and the specific growth rate (SGR) which were higher in early restricted animals (P < .001 and P < .05, respectively). Final body weight, SGR, gained yield and FCR were also significantly modi-

The plasma metabolites (glucose, triglycerides and lactate) were measured at the end of the dietary challenge, 3 h after the last meal (Table 6). The significant effects of the diets were found for the glycemia and the triglycerides showing higher glycemia and lower triglyceridemia in shrimp fed the P43 diet. Only plasma lactate was dependent of the early stimulus with higher levels of plasma lactate in early stimulated shrimp (P < .03). An interaction between the stimulus × dietary challenge (P = .02) was observed for the plasma glucose: the highest plasma glucose level was found in shrimp following the RESP43 treatment (1.31 ± 0.73 mM) whereas no variation was detected in shrimp under the CTL group whatever are the diets.

We also analyzed the mRNA levels from hepatopancreas (Table 7) and muscle (Table 8) tissues 3 h after the last meal. We found several statistical differences for mRNA levels for metabolic genes extracted from hepatopancreas (Table 7). Firstly, the effects of the diets were observed for preamylase, gdh, fbp and pepck gene expressions: mRNA levels for these genes were increased with higher levels of dietary proteins. Secondly, 8 genes were strongly affected by the early stimulus; decreased mRNA levels for the digestive enzymes (lipase, preamylase, and trypsin), amino acid metabolism (gs), energy metabolism (cox VI b) and glucose metabolism (lglut 1, lglut 2 and pk). Finally, only 2 genes (cox VI c and lglut1) shown interactions between stimulus and diets: indeed, lglut 1 was down-regulated linked to the increased of dietary protein on the RES group.

By contrast to the hepatopancreas, no significant differences about mRNA levels for all the genes coding for lipid, amino acid, energy and glucose metabolisms were found in muscle (Table 8).

3.3. Was it possible to detect in the hepatopancreas before the challenge the molecular effects linked to the stimulus history?

We analyzed the mRNA levels for the metabolic genes in hepatopancreas extracted from animals before the dietary challenge (50 days of rearing, i.e., the 3rd sampling in Fig. 1): no significant differences were detected in any of the analyzed genes (Table 9).
4. Discussion

Studies on metabolic programming in mammals reveal that adverse environmental conditions (including nutrition) during critical stages on the early development may have long-term effect on the metabolism or physiology in relation to some diseases (Burdge and Lillycrop, 2010; Devaskar and Thamotharan, 2007; Lucas, 1998; Metges et al., 2014; Patel and Srinivasan, 2002). Based on the concept of metabolic programming, the purpose of this study was to evaluate the effect of the early feed restriction on the metabolic programming in the whiteleg shrimp, *L. vannamei*. This was based on the fact that it is well known that an early nutritional restriction could act as a strong trigger of metabolic adaptation in mammals (Barker and Osmond, 1986; Lucas, 1998; Spencer, 2012). Moreover, this stimulus could be a simple tool to be applied in commercial shrimp hatcheries. Our previous study on the ontogenesis of the metabolic genes suggested a window of the early shrimp larval development (i.e., the early post-larval stage) could be the optimal period to test this concept (Lage et al., 2017). In the present study, we confirm that a high molecular plasticity occurs between the PL1 and PL4 (CTL) stages, as reflected by the differences in the gene expressions for many of the metabolic genes.

Feed restriction of post-larval shrimps is possible to test the programming concept.

Among the different factors to be selected for the early stimuli, the feed restriction was largely studied in animals (Burdge and Lillycrop, 2010; Pittman et al., 2013; Skinner et al., 2010). Feeding level was decreased up to 30% for shrimp post-larvae during three days for the stimulus group compared to the control group. To be able to test the programming concept in animals, it was very important to avoid any selection of the animals. Although it was not possible to evaluate shrimp growth performance just after the short stimulus, measurements carried out at day 12 (corresponding to what is generally done in commercial hatcheries) showed no differences in survival between the two groups (around 50%, as previously observed by Gallardo et al., 2013).

No modification of the mRNA levels of the biomarkers for digestion and intermediary metabolism has been detected at the end of the stimulus. Exception was for the trypsin (gene coding for the protein di-gestion) which was 2 times-fold down-expressed in the RES group. This finding can be related to the fact that, based on the dietary composition (Table 2), the early feed restriction reflects mainly a strong decrease in protein intake. Indeed, it is well known that trypsin activities of *L. vannamei* respond to the levels of dietary crude proteins (Carrillo-Farnés et al., 2007; Lee et al., 1984; Puello-Cruz et al., 2002; Wei et al., 2014). The absence of strong metabolic reactions could be due to the relatively short duration of the stimulus as previously observed in rainbow trout first fed only three days (Geurden et al., 2007).

Nutritional regulation of growth performance and mRNA levels for digestion/metabolism enzymes in juvenile shrimps.

At the end of the dietary challenge phase, growth performances of shrimps were affected by the dietary treatments. Indeed, shrimp fed with the lowest level of dietary proteins had the lowest BWB, SGR, yield and higher FCR. This can be explained by the relatively high protein requirement reported for *L. vannamei* (between 30% and 36%) and the fact that a high digestible protein content (40%) is known to improve growth performance of juvenile shrimps (Kureshy and Davis, 2002; Li et al., 2008, 2017). Here, the increasing the protein/carbohydrate ratio was associated with higher gdh mRNA levels and higher gluconeogenic mRNA levels (*fhp* and *pepk*). These data confirm that *L. vannamei* can adapt well its metabolism relative to the levels of CBH and the dietary protein content, by increasing enzyme activities of amino acid catabo-lism (*gdh*) and gluconeogenesis (*pepk*) as previously shown (Li et al., 2010).
Early feed restriction is associated with modifications of the growth performance and of specific mRNA levels in hepatopancreas in juvenile shrimp: a proof of the existence of metabolic programming.

After the end of the dietary challenge, we observed changes in shrimp growth performance as well as for the mRNA levels of genes in hepatopancreas-tissue linked to the early feed restriction. Indeed, shrimp with early feed restriction history had higher final body weight and SGR but also higher plasma lactate (may be due to higher anaerobic glycolysis (Soñanez-Organis et al., 2010; Soñanez-Organis et al., 2012) and lower mRNA levels for a lot of metabolic and digestive genes in the hepatopancreas.

Regarding the molecular analysis in hepatopancreas, decreased expression of the genes coding for proteins in energy (mitochondrial) metabolism (cox VI c), glucose transport -metabolism (lgat1, lgat2, pk) and glutamine synthetase (gs). Moreover, decreases of the genes coding lipid (lipase), carbohydrate (preamylose) and protein (trypsin) digestion has been also observed. The link between the lower levels of mRNA levels for all these actors and the higher growth performance and/or plasma lactate in the restricted group is not well defined and requires further studies. However, several epidemiological studies revealed previously that Intrauterine Growth Restriction (IUGR) and low birth weight related with maternal undernutrition (i.e., equivalent to a feed restriction process) are correlated with the increase in the risk of non-communicable diseases or metabolic syndrome (type II diabetes, hypertension and cardiovascular disease) on the adulthood with deregulation of glucose homeostasis and lipid metabolism (Duque-Guármara and Ozanne, 2013; Cluckman et al., 2005; Hales and Barker, 2001; Ozanne and Hales, 1999; Patel et al., 2009; Spencer, 2012). Here, as in mammals, our data link feed restriction at early life with modifications of hepatohepatic metabolic in adult shrimp.

In contrast, mRNA levels of metabolic genes in muscle were not affected by the early stimulus, suggesting this tissue is not a target for molecular programming in our study. This is different to previous studies of metabolic programming (in fish) which suggested that muscle was more susceptible for the programming than liver (Geurden et al., 2014; Panserat et al., 2017).

**Are there interactions between diets and nutritional history in juvenile shrimp?**

A few number of interactions (for the FBW, glycemia and only two genes) between early stimulus and diets have been observed in our study. Indeed, the FBW, glycemia and only two genes) between early stimulus and diets have been observed in our study. These data could suggest that only feed-restricted group was able to adapt for more efficient transport of glucose and use it as energy source associated with a better growth performance. Further studies are however needed to conclude definitively about these interactions.

---

### Table 4

<table>
<thead>
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<th>Target genes</th>
<th>CTL</th>
<th>RES</th>
<th>P-value</th>
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<tr>
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<td>Lipase</td>
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<tr>
<td>gdh</td>
<td>0.41 ± 0.30</td>
<td>0.38 ± 0.07</td>
<td>0.548</td>
</tr>
<tr>
<td>Energy metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>atpase a</td>
<td>0.45 ± 0.31</td>
<td>0.49 ± 0.14</td>
<td>0.429</td>
</tr>
<tr>
<td>atpase b</td>
<td>0.58 ± 0.30</td>
<td>0.46 ± 0.12</td>
<td>0.662</td>
</tr>
<tr>
<td>cok VI a</td>
<td>0.56 ± 0.37</td>
<td>0.50 ± 0.10</td>
<td>0.931</td>
</tr>
<tr>
<td>cok VI b</td>
<td>0.70 ± 0.51</td>
<td>0.38 ± 0.16</td>
<td>0.286</td>
</tr>
<tr>
<td>cok VI c</td>
<td>0.51 ± 0.34</td>
<td>0.46 ± 0.10</td>
<td>0.792</td>
</tr>
<tr>
<td>Glucose transport and metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lgat1</td>
<td>0.44 ± 0.24</td>
<td>0.43 ± 0.07</td>
<td>0.931</td>
</tr>
<tr>
<td>lgat2</td>
<td>0.37 ± 0.30</td>
<td>0.40 ± 0.06</td>
<td>0.792</td>
</tr>
<tr>
<td>ldh</td>
<td>0.45 ± 0.39</td>
<td>0.42 ± 0.11</td>
<td>1.000</td>
</tr>
<tr>
<td>pk</td>
<td>0.65 ± 0.22</td>
<td>0.43 ± 0.06</td>
<td>0.190</td>
</tr>
<tr>
<td>hck</td>
<td>0.42 ± 0.19</td>
<td>0.45 ± 0.07</td>
<td>0.951</td>
</tr>
<tr>
<td>fbp</td>
<td>0.46 ± 0.27</td>
<td>0.52 ± 0.19</td>
<td>0.662</td>
</tr>
<tr>
<td>pepck</td>
<td>0.36 ± 0.16</td>
<td>0.32 ± 0.06</td>
<td>0.792</td>
</tr>
</tbody>
</table>

**Table 5**

<table>
<thead>
<tr>
<th>Performance</th>
<th>CTL P30</th>
<th>CTL P37</th>
<th>CTL P43</th>
<th>RES P30</th>
<th>RES P37</th>
<th>RES P43</th>
<th>P-value of Two-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>0.91 ± 0.03</td>
<td>0.90 ± 0.02</td>
<td>0.90 ± 0.02</td>
<td>0.89 ± 0.02</td>
<td>0.89 ± 0.02</td>
<td>0.89 ± 0.03</td>
<td>0.061 ± 0.03</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>8.68 ± 1.10</td>
<td>8.94 ± 1.36</td>
<td>8.73 ± 1.07</td>
<td>8.47 ± 0.88</td>
<td>9.11 ± 1.35</td>
<td>9.37 ± 1.16</td>
<td>9.14 ± 1.26</td>
</tr>
<tr>
<td>Final survival (%)</td>
<td>96.07 ± 4.26</td>
<td>98.21 ± 3.09</td>
<td>97.14 ± 2.04</td>
<td>97.50 ± 2.99</td>
<td>95.71 ± 2.71</td>
<td>95.71 ± 2.71</td>
<td>95.71 ± 2.71</td>
</tr>
<tr>
<td>SGR (% day)</td>
<td>3.22 ± 0.06</td>
<td>3.27 ± 0.16</td>
<td>3.24 ± 0.04</td>
<td>3.22 ± 0.03</td>
<td>3.32 ± 0.04</td>
<td>3.37 ± 0.06</td>
<td>3.37 ± 0.06</td>
</tr>
<tr>
<td>Gained yield (g/m²)</td>
<td>250 ± 8.13</td>
<td>244 ± 27.20</td>
<td>234 ± 14.42</td>
<td>228 ± 11.27</td>
<td>247 ± 8.56</td>
<td>250 ± 10.73</td>
<td>250 ± 10.73</td>
</tr>
<tr>
<td>AFI (g of feed shrimp⁻¹)</td>
<td>10.38 ± 0.26</td>
<td>10.53 ± 0.47</td>
<td>10.28 ± 0.11</td>
<td>10.33 ± 0.24</td>
<td>10.45 ± 0.31</td>
<td>10.52 ± 0.21</td>
<td>10.52 ± 0.21</td>
</tr>
<tr>
<td>FCR</td>
<td>1.24 ± 0.02</td>
<td>1.20 ± 0.07</td>
<td>1.21 ± 0.06</td>
<td>1.25 ± 0.05</td>
<td>1.18 ± 0.02</td>
<td>1.17 ± 0.03</td>
<td>1.17 ± 0.03</td>
</tr>
</tbody>
</table>

Data represent the means ± SD of five replicate tanks. Statistical differences were evaluated by two-way ANOVA. When stimuli × diet interactions were significant, means of all treatments were compared by Tukey’s HSD test. Differences were considered statistically significant when P < 0.05. Lowercase letters indicate differences between dietary treatments.
Table 6
Effect of the early feed restriction and the dietary challenge on postprandial plasma metabolites on Litopenaeus vannamei exposed to a 10-weeks challenge diet, 3 h after the last meal.

<table>
<thead>
<tr>
<th>Plasma metabolites (mmol L⁻¹)</th>
<th>CTL P30</th>
<th>CTL P37</th>
<th>CTL P43</th>
<th>RES P30</th>
<th>RES P37</th>
<th>RES P43</th>
<th>P-value of Two-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.71 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.99 ± 0.29&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.76 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.65 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.31 ± 0.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.892</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.82 ± 0.40</td>
<td>0.92 ± 0.83</td>
<td>0.78 ± 0.36</td>
<td>1.02 ± 0.36</td>
<td>1.66 ± 1.03</td>
<td>1.03 ± 0.56</td>
<td>0.029</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.31 ± 0.09</td>
<td>0.31 ± 0.09</td>
<td>0.25 ± 0.09</td>
<td>0.29 ± 0.08</td>
<td>0.29 ± 0.08</td>
<td>0.23 ± 0.04</td>
<td>0.434</td>
</tr>
</tbody>
</table>

CTL: control group (no feed restricted group). RES: restricted group (feed restricted group). Data were presented as mean ± SD (n = 9; plasmas per condition). Statistical differences were evaluated by two-way ANOVA followed by a Tukey’s HSD test post hoc to identify the significant interaction between stimulus × diet; differences are statistically significant when p < .05. Different letters indicate statistically difference between groups (Stimulus and Diet).

Table 7
Effect of the early feed restriction and the dietary challenge on the mRNA levels in hepatopancreas (ef1a was used as reference gene) of the genes related with the digestive, intermediary and energy metabolism of the Litopenaeus vannamei, extracted from the hepatopancreas-tissue after a 70 days diet challenge.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>CTL P30</th>
<th>CTL P37</th>
<th>CTL P43</th>
<th>RES P30</th>
<th>RES P37</th>
<th>RES P43</th>
<th>P-value of Two-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Digestion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hpse</td>
<td>1.07 ± 0.35</td>
<td>1.18 ± 0.38</td>
<td>1.37 ± 0.71</td>
<td>0.92 ± 0.48</td>
<td>0.73 ± 0.46</td>
<td>0.61 ± 0.10</td>
<td>0.009</td>
</tr>
<tr>
<td>peptd A</td>
<td>1.09 ± 0.27</td>
<td>0.85 ± 0.06</td>
<td>1.33 ± 0.35</td>
<td>0.77 ± 0.29</td>
<td>0.90 ± 0.42</td>
<td>1.04 ± 0.21</td>
<td>0.074</td>
</tr>
<tr>
<td><strong>Lipid metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fas</td>
<td>0.62 ± 0.45</td>
<td>0.84 ± 1.03</td>
<td>1.80 ± 1.49</td>
<td>0.54 ± 0.35</td>
<td>0.91 ± 0.82</td>
<td>1.03 ± 0.68</td>
<td>0.421</td>
</tr>
<tr>
<td><strong>Amino acids metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gs</td>
<td>1.27 ± 0.26</td>
<td>1.10 ± 0.15</td>
<td>1.06 ± 0.15</td>
<td>1.02 ± 0.23</td>
<td>0.88 ± 0.38</td>
<td>1.17 ± 0.26</td>
<td>0.178</td>
</tr>
<tr>
<td><strong>Energy metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>atpase a</td>
<td>0.93 ± 0.11</td>
<td>0.83 ± 0.13</td>
<td>0.98 ± 0.12</td>
<td>0.84 ± 0.24</td>
<td>0.81 ± 0.37</td>
<td>1.03 ± 0.16</td>
<td>0.812</td>
</tr>
<tr>
<td>atpase b</td>
<td>1.10 ± 0.15</td>
<td>0.97 ± 0.12</td>
<td>1.13 ± 0.36</td>
<td>1.05 ± 0.31</td>
<td>0.89 ± 0.36</td>
<td>1.13 ± 0.23</td>
<td>0.633</td>
</tr>
<tr>
<td>cox VI a</td>
<td>0.81 ± 0.20</td>
<td>0.77 ± 0.31</td>
<td>0.91 ± 0.03</td>
<td>0.82 ± 0.02</td>
<td>0.77 ± 0.30</td>
<td>1.02 ± 0.23</td>
<td>0.178</td>
</tr>
<tr>
<td>cox VI c</td>
<td>1.27 ± 0.24</td>
<td>1.06 ± 0.17</td>
<td>1.08 ± 0.22</td>
<td>0.84 ± 0.19</td>
<td>0.96 ± 0.39</td>
<td>1.35 ± 0.36</td>
<td>0.369</td>
</tr>
<tr>
<td><strong>Glucose metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bgal 1</td>
<td>0.59 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28 ± 0.11&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.04 ± 0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.20 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.36 ± 0.27&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.26 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.022</td>
</tr>
<tr>
<td>bgal 2</td>
<td>1.49 ± 0.36</td>
<td>0.97 ± 0.24</td>
<td>1.57 ± 0.47</td>
<td>1.18 ± 0.58</td>
<td>0.76 ± 0.38</td>
<td>0.64 ± 0.76</td>
<td>0.008</td>
</tr>
<tr>
<td>idh</td>
<td>0.76 ± 0.40</td>
<td>1.74 ± 1.34</td>
<td>1.08 ± 1.28</td>
<td>1.24 ± 1.29</td>
<td>0.40 ± 0.19</td>
<td>0.81 ± 0.46</td>
<td>0.231</td>
</tr>
<tr>
<td>pk</td>
<td>1.46 ± 0.29</td>
<td>1.06 ± 0.37</td>
<td>1.26 ± 0.41</td>
<td>0.85 ± 0.30</td>
<td>0.77 ± 0.27</td>
<td>0.79 ± 0.13</td>
<td>0.151</td>
</tr>
<tr>
<td>hcx</td>
<td>1.22 ± 0.23</td>
<td>1.11 ± 0.49</td>
<td>1.34 ± 0.38</td>
<td>1.34 ± 0.39</td>
<td>0.80 ± 0.36</td>
<td>1.10 ± 0.21</td>
<td>0.256</td>
</tr>
<tr>
<td>fbp</td>
<td>0.87 ± 0.17</td>
<td>0.98 ± 0.30</td>
<td>1.33 ± 0.34</td>
<td>0.91 ± 0.40</td>
<td>0.81 ± 0.40</td>
<td>1.13 ± 0.21</td>
<td>0.307</td>
</tr>
<tr>
<td>pepck</td>
<td>0.80 ± 0.57</td>
<td>0.86 ± 0.25</td>
<td>1.76 ± 1.28</td>
<td>0.86 ± 0.34</td>
<td>0.71 ± 0.26</td>
<td>1.42 ± 0.96</td>
<td>0.579</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD (n = 6 hepatopancreas per group). CTL: control group (no feed restricted group). RES: restricted group (feed restricted group). Statistical differences were evaluated by two-way ANOVA (P < .05) followed by a Tukey's HSD test post hoc to identify the significant interaction between stimulus × diet; differences are statistically significant when p < .05. Different letters indicate statistically difference between groups.

5. Conclusion
This study was the first one to demonstrate the programming concept in shrimp. Early feed restriction is associated with modification of gene expression involved in metabolism. These data are promising for the advancement of novel nutritional strategies based on programming, including for example the optimisation of the development of low protein diets for marine shrimp. Other types of early stimuli (using water salinity, temperature, nutrition) in other context (new developmental window, new duration) must be further studied in the future.

Statement of relevances
Our study aimed at characterizing for the first time the possibility of a metabolic programming in Litopenaeus vannamei. We submitted the shrimps to an early feed restriction stimulus at the post-larvae stage during 3 days (RES group). After a common period of rearing (47 days), the shrimps (CTL and RES) were challenged during 70 days with 3 diets containing different levels of proteins. Higher levels of proteins were associated to higher growth performance, higher gdh mRNA level (amino acid catabolism) and higher fbp-pepck mRNA levels (gluconeogenesis) in hepatopancreas. Interestingly, 8 genes (mainly involved in glucose metabolism and digestion) were also differentially expressed in hepatopancreas of shrimps linked to the early stimulus. In conclusion, our study is the first one to prove the existence of programming linked to nutritional stimulus at the post-larvae stage.

Acknowledgments
We acknowledge ANRT and CNPq for funding Luis Paulo Araujo Lage (CIFRE-Brasil/CNPq - GDE – France/Number 217420/2014-1). All the experiments were funded by Neovia and LABOMAR's marine aquaculture experimental station. We thank LABOMAR staff for their daily support during shrimp rearing. We also thank Daniel Arana Braidi for his assistance with hatchery and nursery information and Melanie Sérusier for her technical help in molecular analyses.
Table 8
Effect of the early feed restriction and the dietary challenge on the mRNA levels in muscle tissue (exogenous luciferase was used as reference gene) of the genes related with the intermediary and energy metabolism of the *Litopenaeus vannamei*, extracted from the muscle-tissue after a 70 days diet challenge.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>CTL P30</th>
<th>CTL P37</th>
<th>CTL P43</th>
<th>RES P30</th>
<th>RES P37</th>
<th>RES P43</th>
<th>P-value of Two-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fas</td>
<td>0.68 ± 0.38</td>
<td>1.39 ± 1.15</td>
<td>1.09 ± 0.87</td>
<td>0.98 ± 1.45</td>
<td>0.87 ± 1.12</td>
<td>1.52 ± 0.73</td>
<td>0.792 ± 0.505 ± 0.485</td>
</tr>
<tr>
<td>Amino acids metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pab</td>
<td>0.77 ± 0.40</td>
<td>0.90 ± 0.42</td>
<td>0.89 ± 0.28</td>
<td>0.92 ± 0.66</td>
<td>1.02 ± 0.39</td>
<td>1.21 ± 0.67</td>
<td>0.228 ± 0.600 ± 0.863</td>
</tr>
<tr>
<td><strong>Energy metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>atpase a</td>
<td>0.95 ± 0.84</td>
<td>1.19 ± 0.57</td>
<td>0.89 ± 0.38</td>
<td>0.95 ± 0.63</td>
<td>0.79 ± 0.36</td>
<td>1.20 ± 0.68</td>
<td>0.933 ± 0.921 ± 0.390</td>
</tr>
<tr>
<td>atpase b</td>
<td>0.97 ± 0.95</td>
<td>1.12 ± 0.62</td>
<td>0.91 ± 0.38</td>
<td>1.01 ± 0.79</td>
<td>0.81 ± 0.32</td>
<td>1.24 ± 0.70</td>
<td>0.904 ± 0.931 ± 0.531</td>
</tr>
<tr>
<td>cox VI a</td>
<td>0.92 ± 0.52</td>
<td>1.25 ± 0.80</td>
<td>0.93 ± 0.42</td>
<td>1.18 ± 0.86</td>
<td>0.82 ± 0.19</td>
<td>1.34 ± 0.84</td>
<td>0.669 ± 0.941 ± 0.290</td>
</tr>
<tr>
<td>cox VI b</td>
<td>0.93 ± 0.51</td>
<td>1.07 ± 0.54</td>
<td>1.04 ± 0.32</td>
<td>1.16 ± 0.54</td>
<td>0.99 ± 0.25</td>
<td>1.39 ± 0.80</td>
<td>0.348 ± 0.662 ± 0.620</td>
</tr>
<tr>
<td>cox VI c</td>
<td>0.97 ± 0.60</td>
<td>1.12 ± 0.56</td>
<td>1.00 ± 0.37</td>
<td>1.25 ± 0.63</td>
<td>0.94 ± 0.16</td>
<td>1.30 ± 0.67</td>
<td>0.456 ± 0.893 ± 0.499</td>
</tr>
<tr>
<td><strong>Glucose metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fruct I</td>
<td>0.69 ± 0.55</td>
<td>1.11 ± 0.50</td>
<td>0.81 ± 0.27</td>
<td>0.86 ± 0.46</td>
<td>0.70 ± 0.33</td>
<td>1.83 ± 1.71</td>
<td>0.326 ± 0.263 ± 0.126</td>
</tr>
<tr>
<td>pk</td>
<td>1.10 ± 0.32</td>
<td>1.15 ± 0.86</td>
<td>0.84 ± 0.46</td>
<td>0.79 ± 0.95</td>
<td>1.25 ± 0.52</td>
<td>1.18 ± 0.90</td>
<td>0.854 ± 0.683 ± 0.538</td>
</tr>
<tr>
<td>ldh</td>
<td>1.22 ± 1.51</td>
<td>1.66 ± 1.15</td>
<td>1.13 ± 0.60</td>
<td>0.74 ± 0.87</td>
<td>0.55 ± 0.31</td>
<td>1.29 ± 0.99</td>
<td>0.186 ± 0.847 ± 0.328</td>
</tr>
<tr>
<td>hex</td>
<td>0.95 ± 0.28</td>
<td>1.16 ± 0.99</td>
<td>0.84 ± 0.56</td>
<td>0.83 ± 1.16</td>
<td>1.31 ± 0.81</td>
<td>1.29 ± 1.11</td>
<td>0.590 ± 0.656 ± 0.738</td>
</tr>
<tr>
<td>f6p</td>
<td>0.92 ± 1.54</td>
<td>1.44 ± 2.16</td>
<td>1.38 ± 0.79</td>
<td>0.39 ± 0.45</td>
<td>0.41 ± 0.26</td>
<td>1.69 ± 1.88</td>
<td>0.411 ± 0.512 ± 0.520</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD (n = 6 muscle per group). CTL: control group. RES: restriction group. Statistical differences were evaluated by two-way ANOVA followed by a Tukey’s HSD test post hoc to identify the significant interaction between stimulus × diet; differences are statistically significant when p < .05.

Table 9
Effect of the early feed restriction (Nutritional History) on the mRNA levels of the genes related with the digestive, intermediary and energy metabolism of the *Litopenaeus vannamei*, extracted from the hepatopancreas tissue before the beginning of the dietary challenge phase (Day 50).

<table>
<thead>
<tr>
<th>Target genes</th>
<th>CTL</th>
<th>RES</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nutritional history</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Digestion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lipase</td>
<td>0.98 ± 0.66</td>
<td>0.98 ± 0.43</td>
<td>0.931</td>
</tr>
<tr>
<td>pepsin</td>
<td>0.89 ± 0.57</td>
<td>0.85 ± 0.35</td>
<td>0.662</td>
</tr>
<tr>
<td>chymotrypsin</td>
<td>1.02 ± 0.63</td>
<td>0.76 ± 0.35</td>
<td>0.931</td>
</tr>
<tr>
<td>trypsin</td>
<td>0.56 ± 0.26</td>
<td>0.71 ± 0.83</td>
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<tr>
<td><strong>Lipid metabolism</strong></td>
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<tr>
<td>fas</td>
<td>1.47 ± 1.21</td>
<td>1.65 ± 0.40</td>
<td>0.309</td>
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<tr>
<td>g6</td>
<td>0.80 ± 0.70</td>
<td>0.54 ± 0.48</td>
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<tr>
<td>gdh</td>
<td>0.79 ± 0.70</td>
<td>0.72 ± 0.32</td>
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<tr>
<td>atpase b</td>
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<tr>
<td>cox VI a</td>
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<td>1.02 ± 0.22</td>
<td>0.609</td>
</tr>
<tr>
<td>cox VI b</td>
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<td>0.78 ± 0.28</td>
<td>0.914</td>
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<tr>
<td>cox VI c</td>
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<tr>
<td>fruct II</td>
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<td>0.69 ± 0.64</td>
<td>0.352</td>
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</table>

Data were presented as mean ± SD (n = 6 hepatopancreas per experimental group). CTL: control group. RES: restriction group. The reference gene ef1a was used to normalize the mRNA levels. After confirming that these data were not normally distributed assessed by Shapiro-Wilk test, statistical differences were evaluated by Wilexon test; differences are statistically significant when p < .05.

References
Balabanian-Masri, M., Pas更有相关数据和信息。


Supplementary data
First set of supplementary data: global DNA methylation analysis in hepatopancreas

The early environmental conditions (nutrition) during the early development may induce persistent adaptation at long-term and the epigenetic modifications could be one of the main mechanisms that is at the origin of the programming. Thus, we also investigated the global DNA methylation from genomic DNA extract from hepatopancreas tissue after the dietary challenge. However, due to the limited number of samples remained after the RNA analysis, we could not evaluate the DNA methylation for all the treatments with the minimum required number of individuals; this is why we analyzed only two pools of the remaining samples corresponding to each of the nutritional histories (RES and CTL). No significant difference was observed after the 70-days diet challenge (data not shown). Even though we must be very prudent with these preliminary data about DNA methylation, it seems that no detectable effect of the nutritional stimulus on the global DNA methylation was detected in hepatopancreas. Further studies about DNA methylation (other approaches to measure DNA methylation, higher number of samples to be studied) must be carried out in the future to conclude about the relation of the nutritional programming in shrimp with the epigenetics marks at the DNA level.

Second set of supplementary data; zootechnical data for the shrimp fed the 4th diet for the dietary challenge

Initially in our experiment, four diets were formulated for the 70-days dietary challenge. However, we decided to not analyse (biochemical, molecular analysis) the animals fed with the 4th diet (35% of CP) for submission of the paper in Aquaculture. Anyway, regarding the growth performance, the early feed restriction affected the final body weight after the challenge phase. Feed restricted group (named RES P35; 9.06 ± 1.22 g) had a significantly higher final body weight compared with the control group (CTL P35; 8.56 ± 1.09 g) (p<0.001). These zootechnical data with the 4th diet suggest one more time that the early feed restriction stimulus was beneficial for programming shrimp to improved the ability of use the protein at the long-term.
Publication 3
4.3 – Nutritional programming of shrimp at the protozoea stage (paper to be submitted)

Summary of the article

Objective

In the present study, we aimed to evaluate the effects of an early feed restriction during the protozoea phase in order to promote long-term modification of the nutrient use in the whiteleg shrimp <i>L. vannamei</i> through the nutritional programming concept.

Experiment and analyses

Animals at nauplii sub-stage 3 (N3) were purchased on a commercial hatchery and transferred to our laboratory. At the protozoea stage (between sub-stage Z1 and Z3) shrimp were feed restricted, 40% lower (RES) than standard level of feeding (CTL) during 4 days. After this early stimulus, all shrimp groups were fed similarly following the BernAqua feeding protocol and reared on the same condition until the end of experiment at the 40<sup>th</sup> day. Zootechnical performances were evaluated at the 25<sup>th</sup> and 40<sup>th</sup> days, which represent respectively the end of hatchery phase and the end of the experimental period. Samples collected at the 5<sup>th</sup> day (end of stimulus) and 25<sup>th</sup> and 40<sup>th</sup> days, were analyzed by RT-qPCR.

Main results

Based on previous studies, the stage of protozoea (first-feeding stage) it is a potential very good candidate to test the nutritional programming, similarly to what has been observed in fish showing high molecular plasticity during the transition between endogenous and exogenous feeding.

At the end of the experimental period, shrimp growth performance was evaluated; no difference between normal feeding shrimp (CTL) and feed-restricted shrimp (RES) for zootechnical parameters (survival, final body weight and number of post-larvae g<sup>−1</sup> or PL g<sup>−1</sup>) were found. The stimulus affected directly the mRNA levels for two genes only i.e. <i>preamylase</i> and <i>lvglut 2</i> which were higher expressed in feed-restricted shrimp. At long-term, higher levels of mRNAs for enzymes coding for glycolysis and ATP synthesis were detected suggesting a possible modification at long term of the metabolism linked to the stimulus at the protozoea stage. Overall, further studies should be performed to better describe the existence of nutritional programming in shrimp.
In conclusion, our data suggest that the early nutritional stimulus at protozoea stage as a strong candidate being able to change permanently the growth performance of *L. vannamei*. Despite only few changes related to the gene expression at the mid-long term were observed, data are promising concerning the nutritional programming. However, further studies are thus needed using the protozoea stage.
The 4-day feeding restriction at the protozoea stage had a little effect at long term on metabolic gene expressions in whiteleg shrimp (*Litopenaeus vannamei*).
Abstract

Based on the “nutritional programming” concept, we evaluated the long term effects of a three days early caloric restriction (40% reduction of feeding compared to a normal feeding level) in whiteleg shrimp protozoea stage. We analyzed long-term programming of shrimp by studying metabolism at the molecular level, through RT-qPCR of key biomarkers (involved in intermediary metabolism and digestion). mRNA levels (extracted from the whole body) were analyzed after the stimulus and after the common rearing period, after 20 and 35 days, respectively. At the end of the experimental period, shrimp growth performance was evaluated; no difference between normal feeding shrimp (CTL) and feed-restricted shrimp (RES) for zootecchnical parameters (survival, final body weight and number of post-larvae g⁻¹ or PL g⁻¹) were found. The stimulus affected directly the mRNA levels for two genes only i.e. preamylase and lvglut 2 which were higher expressed in feed-restricted shrimp. At long-term, higher levels of mRNAs for enzymes coding for glycolysis and ATP synthesis were detected suggesting a possible modification at long term of the metabolism linked to the stimulus at the protozoea stage. Overall, further studies should be performed to better describe the existence of nutritional programming in shrimp.

Key words: whiteleg shrimp, feed restriction, gene expression, metabolism, digestion, programming
Introduction

Considering all the sectors of animal food production, aquaculture have the higher growth rate and represent more than 50% of the worldwide fish consumption, since global wild fish capture have stagnated over the last 30 years. Marine shrimp production, despite its small volume production representing less than 10% of volume the aquaculture production, accounts for 22.6% of the total aquaculture value (FAO, 2016). However, there is a need to improve the shrimp productions to ensure its sustainability.

Even though several studies shown that whiteleg shrimp *Litopenaeus vannamei* can be raised with the almost complete replacement of fishmeal (FM) by plant protein mainly soybean meal (Sabry-Neto et al., 2016; Sookying et al., 2013; Suárez et al., 2009), commercial diets used for the rearing of penaeid shrimp rely mainly on fishmeal (FM) and fish oil (FO) as the main source of dietary protein and lipid, respectively. Soybean meal compared with FM, has lower nutrient digestibility, non-adequate amino acids profile and methionine deficiency, anti-nutritional factors and poor attractability (Gatlin et al., 2007; Tacon and Metian, 2008). However, FM and FO are produced by the capture of wild fish which are actually over exploited. Therefore, there is ongoing efforts to develop alternative diets with a decrease of FM and FO ingredients by incorporating plant-based ingredients or byproducts obtained from agriculture and animal (FAO, 2016; Naylor et al., 2009; Nunes et al., 2014). Albeit up to now, the total replacement of FM by alternative ingredients remains a difficult task, it is feasible with the supplementation of essential amino acids and fatty acids (Nunes et al., 2014; Tacon and Metian, 2008).

Moreover, the decrease of the level of dietary proteins by inclusion of carbohydrates is also not easy (Cuzon et al., 2004; Gatlin et al., 2007; NRC, 2011; Wang et al., 2016). Research of new nutritional strategies to improve the use of alternative diets is an important objective in shrimp nutrition.

The early development of animals is characterized by a period of high molecular plasticity. The concept of nutritional programming refers to the early events (environmental factors i.e.; nutrition, toxic exposure, oxygen, temperature) performed either on the prenatal or postnatal periods which may have a persistent long-term effect either on metabolism or/and on physiology (Duque-Guimarães and Ozanne, 2013; Gluckman et al., 2005; Lucas, 1998; Patel et al., 2009; Patel and Srinivasan, 2002). Recently, studies on the metabolic programing in several species of fish have been successfully performed mainly focused on the early feeding
(Balasubramanian et al., 2016; Fang et al., 2014; Panserat et al., 2017; Rocha et al., 2016a, 2016b; Vagner et al., 2009).

Nevertheless, nutritional programming studies on the whiteleg shrimp *Litopenaues vannamei* are very scarce. Recently, Lage et al (2017) mapped the genes involved in intermediary metabolism at the embryonic and larval development suggesting the existence of two developmental windows (protozoea stage and post-larvae stage) which could be optimal to test that concept. Indeed, the first study about nutritional programming through feed restriction (well known to be a major factor of metabolic programming in mammals (Barker and Osmond, 1986; Lucas, 1998; Spencer, 2012)) was performed in shrimp during the post-larvae stage. Lage and colleagues (2018), observed that an early nutritional stimulus improve the growth performance associated with altered mRNA levels for genes related to digestion, amino acids, energy and glucose metabolism.

The aim of the present study was to evaluate the effect of an early feed restriction (40% lower than standard level of feeding) during the protozoea phase (between sub-stages Z1 and Z3, the second developmental window with high level of molecular plasticity (Lage et al., 2017)) in order to modify the nutrient use in whiteleg shrimp *L. vannamei* through nutritional programming.

**Material and methods**

**Rearing conditions and reception of Litopenaeus vannamei larvae**

Salty water was pumped from the Pacoti Estuary (03150001.5500S and 38125022.7400W) and stocked on two reservoirs, 10 and 5 m³. By this time, water salinity was adjusted equally to the hatchery conditions (30 ups) adding fresh/tap water, then disinfected with chlorine (0.02 g/L) and sand-filtered during 48h to remove big size particles and neutralize the chlorine. To assure that there was no remain chlorine on the water system, a chlorine test (Labcon CloroTest, Alcon) was performed and finally sodium thiosulphate (5 ppm) was added as a chlorine neutralizer.

The system was characterized by 16 rectangular tanks 61L total volume and dimensions 31.0 x 35.5 x 55.5 cm (height x width x length), operate on clear water recirculation system by 2.0-hp water pump and sand filter (particles larger than 50 µm) connected to two reservoirs tanks of 10 and 5 m³. In all rearing tanks, there is a continuous aeration provided by two 2.0-hp air blowers and one air diffusers per tank.
One hundred and twenty thousand *Litopenaeus vannamei* N3 naupli sub-stage 3 were purchased on a commercial hatchery (CELM Aquicultura, Aracati S/A – Ceara, Brazil) and transported on plastic bags contain dissolved oxygen to the Laboratory of Aquatic Animal Nutrition LANOA (3°50′01.55″S and 38°25′22.74″W) located in Eusebio, Brazil. Salty water from the Pacoti Estuary was pumped to the Laboratory of Aquatic Animal Nutrition LANOA (Eusebio – Ceara, Brasil) and stocked on two reservoirs of 10 and 5 m³ (totalizing 15 m³). By this time, water salinity was adjusted at the same level than at the hatchery salinity (30 ups) by adding fresh/tap water, disinfected with chlorine (0.02 g/L) and sand-filtered for 24 hours to remove big size particles posteriorly, filtered by carbon filter.

**Shrimp rearing and feeding**

The Figure 1 describes the experimental design. To avoid stress of high temperature 7,500 naupli N3 were stoked during the nightfall period to the experimental units (initial density of 125 naupli.L⁻¹). A monophasic system was used on the experiment; hatchery and nursery phases were performed on the same tank.

**Stimulus protocol through feed restriction**

The early nutritional stimulus started when larvae reached the protozoea Z1 sub-stage 1. Animals were then divided into one experimental group (RES) and a control group (CTL) with 8 replicates tanks for each. Protozoea shrimp were fed following a commercial hatchery feeding guide (BernAqua NV, Olen, Belgium). The CTL group were fed following normal feeding rates while the treatment (RES) was raised under a 40% restriction of the feeding level (Table 1). During all the experiment, no disinfectant or probiotics were used. The microalgae *Thalassiosira* spp was used as live feed from the late naupli N5/early protozoea Z1 sub-stages to post-larvae PL3 and *Artemia* was totally replaced by Vitellus (BernAqua). Chlorine (20 ppm) was utilized to filtered salt water sterilization. Afterwards, chlorine was neutralized with sodium thiosulphate (5 ppm).

After the early stimulus (4 days) all the experimental groups were fed similarly, following the BernAqua feeding protocol, until the end of the experiment at the 40th day.
Shrimp sampling

Shrimp larvae were sampled three times after the early stimulus (Day 5), at the end of hatchery phase (Day 25) and at the end of the experiment (Day 40) (Figure 1). For each samplings, shrimp larvae were washed with sterile salt water, and dried with an absorbent paper. Samples were preserved on a RNA Stabilization Solution (RNA Later Sigma®), 100 mg tissue /1 mL RNA Later, immediately immersed on this solution and kept under -20°C.

Total RNA extractions and relative quantification of mRNA levels

Biological material was collected with a sterile mesh (100 µm) and then dried with an absorbent paper to remove the maximum amount possible of the RNA Later product. Subsequently, the tissue was weighed and immediately immersed on a Trizol solution. RNA extractions, from whole body, were performed using the reagent Trizol® (100 mg sample / 1 mL Trizol) and following the recommendations of the company. The RNA concentrations were determined using the spectrophotometer NanoDrop 2000 whereas the RNA qualities were determined after migration on a 1% agarose gel electrophoresis.

mRNA levels were determined using the real-time RT-PCR (n=6 RNA samples per treatment). The mRNA levels of 19 genes coding for proteins involved in macronutrient digestion, nutritional metabolism (amino acid, lipid and glucose), glucose transport and mitochondrial metabolism were quantified using shrimp specific primers. The primer sequences used in the real-time RT-PCR assays are the same than those used in our previous study, recently published (Lage et al., 2017).

For the RT-qPCR, an amount of 1µg RNA was reverse transcribed to cDNA with SuperScript III RNase H-Reverse Transcriptase Kit (Invitrogen) with random primers (Promega, USA). Real-time PCR was performed in the LightCycler 480 (ROCHE, Hercules, CA, USA). Quantitative PCR (Q-PCR) analyses for gene expressions were performed using a reaction mix of 6 µL per sample containing 2 µL of the RT produce (diluted cDNA), 0.24 µL of each primer (10 µmol/L), 3 µL Light Cycler 480 SYBR® Green and 0.54 µL DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany) as previously described (Dai et al., 2014). Melting curves were systematically monitored (temperature gradient at 0.5 °C/10 s from 55 to 94 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each q-PCR run included duplicates of samples (reverse transcription) and negative controls (wells without reverse transcriptase, mRNA and cDNA). Relative quantification of target gene expression was
performed using the ΔCT method (Pfaffl, 2001). The relative quantification of mRNA levels of target genes were normalized with the *Litopenaeus vannamei* elongation factor 1-alpha (ef1α) gene. In all cases, PCR efficiency (E) was measured by the slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency values ranged between 1.8 and 2.2.

**Statistical Analyses**

Statistical analyses were carried out using R software (v.3.1.0)/R Commander Package, for the zootechnical performance, molecular and biochemical analyses. Prior to statistical analyses, assumption of data normality and homogeneity of variances were assessed using the Shapiro-Wilk test and Levene’s test, respectively.

Zootechnical parameters i.e. survival (%), final body weight (g) and PL.g⁻¹ (number of PL per gram) were evaluated by using a Student’s t-test. Data are represented as mean ± SD (n=8 tanks per experimental group), the data are significantly different when the P<0.05. For the mRNA levels analysis (n=6 samples per experimental group) after the stimulus and at the end of nursery and hatchery phases, statistical differences were evaluated by Wilcoxon test; since data did not follow a normal distribution. All the experimental data are presented as mean ± SD. The data are significantly different when the P<0.05.

**Results and discussion**

Protozoea in shrimp is the first phase related with the exogenous feeding. As in fish (Mennigen *et al.*, 2013), the first feeding stage (i.e. the protozoea stage) is characterized by a strong plasticity of the metabolism at the molecular level (Lage *et al.*, 2017; Mennigen *et al.*, 2013).

*Early nutritional feed restricted stimulus in protozoea shrimp: low impacts on the mRNA levels coding for metabolic and digestive proteins*

The early stimulus by using a feed restriction protocol on the protozoea shrimp did not result in a decrease of the shrimp survival at the end of hatchery phase. Based on our previous study (Lage *et al.*, 2017) we evaluated that the developmental stage of protozoea (protozoea sub-stages Z1, Z2 and Z3) could be the optimal window to perform a nutritional programming
regarding the molecular plasticity. Thus, we choose the timing between the sub-stage Z1 and the sub-stage Z3 (4 days) to be the caloric restriction stimulus.

To test the direct effect of the early nutritional stimulus, comparison between the normal feed rate (CTL) and the one with 40% lower than normal feeding allowance (RES) (Figure 1 and Table 1), we analyzed the mRNA levels for the genes coding for the digestion, intermediary and energetic metabolism at the whole body level. Only preamylase and glucose transporter 2 (lvglut 2) mRNA levels were different between the conditions, where both genes were upregulated 1.47 and 1.89 times respectively in restricted group, \( P<0.05; \) Table 2. The mRNA levels of chymotrypsin were not detected due its very low level of expression as previously shown (Lage et al., 2017). Our molecular data do not suggest a strong effect of the stimulus on the protozoea shrimp. This could be explained by: (i) the stage of development used for the present stimulus; (ii) the level of restriction which is relatively weak (40%). Indeed, L. vannamei, is a crustacean for which, during the early development, the specie pass through typical metamorphosis (embryo, nauplii, protozoea, mysis and, post-larvae steps) before becoming adults (Dall et al., 1990). During this process and especially during the protozoea phase, the small size and fragility of the animals, is evident that a stronger stimulus was not possible in order to avoid any decrease of survivals. Moreover, despite the role of dietary proteins (and their digestion) on the larval development (Carrillo-Farnés et al., 2007; Le Vay et al., 2001; Puello-Cruz et al., 2002), the present feeding restriction did not altered the mRNA levels of trypsin between CTL and RES animals. By contrast, surprisingly, preamylase mRNA levels were higher in RES animals. Previous studies reported expression of preamylase gene at protozoea stages (Lage et al., 2017; Wei et al., 2014b), however they show a strong differences between the different protozoea sub-stages (high expression in Z1 and Z3 stages and low in Z2 and M1 stage) (Lage et al., 2017). Our data suggest that the RES animals could be a slight modification of their development kinetic.

If we compared the effects of the feed restriction stimulus with the one we did in the post-larvae stage (Lage et al. 2018), only trypsin gene expression was affected by the feed restriction in this case probably linked to the decrease of protein intake. This is not the case in the present study. However, the post-larvae stimulus was stronger (70%). The difference of developmental window that was performed the stimulus as well as the strength of the stimulus could explain the differences of the reactions of the biomarkers that we observed between the two studies.
Test of the existence of the programming through common rearing period (35 days): no modification of the shrimp performance and slight alterations of some mRNA levels coding for energy and glycolytic proteins

After the stimulus period (4 days), RES and CTL animals were reared under the same conditions until the end of the experiment at the 40th day (Figure 1) in order to test the long-term effect (programming effect) of the early stimulus. We selected two samplings for our analysis; the first one at the end of the hatchery phase (day 25th) and the second one at the end of the experiment (during the nursery phase) at day 40th. Shrimp performance was analyzed at the 25th day as shown in Table 3. No significant differences between shrimp issue from the CTL and the RES groups were observed on final body weight and number of post-larvae g\(^{-1}\) or PL g\(^{-1}\). No statistical differences on the mRNA levels were observed for all the genes, except for the trypsin gene that was slightly up regulated in RES group (Table 4). Shrimp performance were also analyzed at the 40th rearing day which correspond to the end of experiment (Table 3). No significant differences between shrimp issue from the control and the stimulus groups were observed on survival, final body weight and number of post-larvae g\(^{-1}\) or PL g\(^{-1}\). The mRNA levels significant differences were observed for the genes coding for enzymes involved in energy metabolism atpase a and b, in anaerobic glycolysis lactate dehydrogenase (ldh) and in aerobic glycolysis pyruvate kinase (pk) and hexokinase (hk) (Table 5). Overall, all the differentially expressed genes were up regulated on the RES group compared to the CTL group except for the hk. We can note that these molecular biomarkers suggest that a metabolic programming could be linked to the early nutritional stimulus at the protozoea phase. However, these differences are relatively weak (low level of statistical significances) and globally different with our first study (Lage et al., 2018). Indeed, an early feeding restriction stimulus on the whiteleg shrimp _L. vannamei_ was used, successfully, for the first time during the post-larvae stage by Lage et al. (2018). A better growth performance for RES shrimp as well as different-expressed biomarkers in relation to digestion and glucose metabolism were observed. The differences between the two programming studies (this one and the one by Lage et al., 2018) could be due to the levels of the restriction (40% versus 70%), the developmental windows used (protozoea versus post-larvae), the duration of the experiment (45 days versus 127 days) and the dietary challenge used in the first study only (with 3 diets).
Conclusion

In conclusion, our data suggest that the early nutritional stimulus at protozoa stage was not able to change permanently and strongly the metabolism and growth performance in shrimp in contrasts to the first study (Lage et al., 2018). Further studies are thus needed to improve our knowledge about the possibility of use of programming for better shrimp growth performance and nutrition in aquaculture.
Legends to the figures

**Figure 1.** Overview of the experimental design from the early nutritional stimulus at the protozoea stage up to the juvenile stage. All the sampling were done as a pool of the whole body of shrimp. §: evaluation of zootechnical performances.
**Table 1:** Stimulus Restriction Feeding Protocol for *L. vannamei* (during 4 days from protozoea Z1 sub-stage up to the Z3 sub-stage). CTL: control group. RES: feed restricted group (40% of reduction of the feeding level of the CTL group).

<table>
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<th>Treatment</th>
<th>Day</th>
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<th>Vitellus Standard ⁴</th>
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<td>4</td>
<td>72</td>
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Proximate composition (%), as is⁵

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<td>Proteins</td>
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<td>Lipids</td>
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<td>Ash</td>
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<tr>
<td>Moisture</td>
<td>8.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

¹Feeding guide based on BernAqua NV (Olen, Belgium) recommendations.
²*Thalassiosira* spp (1000 cel mL⁻¹).
³(BernAqua NV, Olen, Belgium). Microcapsulated larval diet. Particle size from 50-100 µm.
⁴(BernAqua NV, Olen, Belgium). Particle size from 50-125 µm.
⁵Product label. Manufacturer guarantee nutrient levels.
⁶NFE, calculated by difference (100 – crude protein – crude fiber – crude fat – ash).
Table 2: Direct effect of the early feed restriction (stimulus) on the mRNA levels (normalized by the reference gene ef1a) of the genes coding digestive, intermediary and energy metabolism measured at the whole body level of L. vannamei protozoea sub-stage 3 (Z3). Data were presented as mean ± SD (n=6 per experimental group). CTL: control group (no feed restricted group). RES: restricted group (feed restricted group). After confirming that these data were not normally distributed assessed by Shapiro-Wilk test, statistical differences were evaluated by Wilcoxon test. Differences were considered statistically significant when $P<0.05$. ND means no detectable gene expression.

<table>
<thead>
<tr>
<th>Target Genes</th>
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<th>CTL</th>
<th>RES</th>
<th>P-value</th>
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<tr>
<td></td>
<td></td>
<td>Means ± SD</td>
<td>Means ± SD</td>
<td></td>
</tr>
<tr>
<td><strong>Digestion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lipase</td>
<td></td>
<td>0.97 ± 0.28</td>
<td>1.17 ± 0.19</td>
<td>0.097</td>
</tr>
<tr>
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<td>0.014</td>
</tr>
<tr>
<td>chymotrypsin</td>
<td></td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>trypsin</td>
<td></td>
<td>0.95 ± 0.34</td>
<td>1.05 ± 0.19</td>
<td>0.620</td>
</tr>
<tr>
<td><strong>Lipid Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fas</td>
<td></td>
<td>1.11 ± 0.55</td>
<td>1.07 ± 0.12</td>
<td>0.128</td>
</tr>
<tr>
<td><strong>Amino Acid Metabolism</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>gs</td>
<td></td>
<td>1.08 ± 0.27</td>
<td>1.13 ± 0.18</td>
<td>0.620</td>
</tr>
<tr>
<td>gdh</td>
<td></td>
<td>0.95 ± 0.26</td>
<td>0.98 ± 0.12</td>
<td>0.534</td>
</tr>
<tr>
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<tr>
<td>atpase a</td>
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<td>1.06 ± 0.15</td>
<td>1.04 ± 0.17</td>
<td>0.620</td>
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<tr>
<td>atpase b</td>
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</tr>
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<tr>
<td>cox VI b</td>
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<td>1.22 ± 0.28</td>
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<td>0.901</td>
</tr>
<tr>
<td>cox VI c</td>
<td></td>
<td>1.19 ± 0.09</td>
<td>1.17 ± 0.28</td>
<td>0.710</td>
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<tr>
<td><strong>Glucose Transport and Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lvglut 1</td>
<td></td>
<td>0.97 ± 0.020</td>
<td>1.04 ± 0.08</td>
<td>0.318</td>
</tr>
<tr>
<td>lvglut 2</td>
<td></td>
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<td>1.13 ± 0.40</td>
<td>0.026</td>
</tr>
<tr>
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</tr>
<tr>
<td>pk</td>
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<td>1.04 ± 0.09</td>
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</tr>
<tr>
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<td>1.01 ± 0.18</td>
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<tr>
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<td>1.11 ± 0.19</td>
<td>0.805</td>
</tr>
<tr>
<td>pepck</td>
<td></td>
<td>1.00 ± 0.34</td>
<td>1.07 ± 0.25</td>
<td>0.731</td>
</tr>
</tbody>
</table>
Table 3: Performance of post-larval *L. vannamei* in the hatchery culture phase, *i.e.*, from N3 stage up to PL12 stage (hatchery) and from N3 stage up to the PL 29 stage (juvenile; nursery). CTL, control group (no feed restriction); RES, feed-restricted group. Juvenile statistical (*n* = 8; mean ± SD) was evaluated by Student t-test (*P*<0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Performance</th>
<th>CTL</th>
<th>RES</th>
<th>P Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatchery</td>
<td>Body Weight (mg)</td>
<td>2.14 ± 0.45</td>
<td>2.44 ± 1.09</td>
<td>0.521</td>
</tr>
<tr>
<td></td>
<td>0.521 PL/g</td>
<td>497.21 ± 87.30</td>
<td>170.06 ± 46.70</td>
<td>0.725</td>
</tr>
<tr>
<td>Nursery</td>
<td>Final Survival (%)</td>
<td>41.00 ± 8.01</td>
<td>39.39 ± 5.98</td>
<td>0.740</td>
</tr>
<tr>
<td></td>
<td>Body Weight (mg)</td>
<td>6.25 ± 0.65</td>
<td>6.40 ± 2.40</td>
<td>0.901</td>
</tr>
<tr>
<td></td>
<td>PL/g</td>
<td>161.43 ± 18.10</td>
<td>170.06 ± 46.70</td>
<td>0.719</td>
</tr>
</tbody>
</table>
**Table 4**: Effect of the early feed restriction (Nutritional History) on the mRNA levels (normalized by the reference gene *ef1a*) of the genes coding digestive, intermediary and energy metabolic enzymes and transporters of the *Litopenaeus vannamei*, extracted from the whole body larvae at the end of hatchery phase (Day 25). Data were presented as mean ± SD (n=6 pools of whole body per experimental group). CTL: control group. RES: restriction group. After confirming that these data were not normally distributed assessed by Shapiro-Wilk test, statistical differences were evaluated by Wilcoxon test. Differences were considered statistically significant when \( P<0.05 \). ND means no detectable.

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Hatchery</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL</td>
<td>RES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Means ± SD</td>
<td>Means ± SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Digestion</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>lipase</em></td>
<td>1.03 ± 0.19</td>
<td>0.94 ± 0.13</td>
<td>0.195</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>preamylase</em></td>
<td>0.95 ± 0.32</td>
<td>0.82 ± 0.22</td>
<td>0.279</td>
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<tr>
<td><em>chymotrypsin</em></td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>trypsin</em></td>
<td>0.95 ± 0.21</td>
<td>1.10 ± 0.10</td>
<td>0.038</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lipid Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>fas</em></td>
<td>0.93 ± 0.21</td>
<td>1.07 ± 0.29</td>
<td>0.328</td>
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<td></td>
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<td><strong>Amino Acid Metabolism</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>gs</em></td>
<td>0.98 ± 0.10</td>
<td>1.00 ± 0.20</td>
<td>0.721</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>gdh</em></td>
<td>0.77 ± 0.07</td>
<td>0.75 ± 0.07</td>
<td>0.462</td>
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<td><strong>Energy Metabolism</strong></td>
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<td></td>
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</tr>
<tr>
<td><em>atpase a</em></td>
<td>0.94 ± 0.08</td>
<td>0.98 ± 0.08</td>
<td>0.382</td>
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<tr>
<td><em>atpase b</em></td>
<td>1.02 ± 0.13</td>
<td>1.04 ± 0.11</td>
<td>0.916</td>
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<tr>
<td><em>cox VI a</em></td>
<td>1.03 ± 0.09</td>
<td>1.04 ± 0.07</td>
<td>0.798</td>
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<tr>
<td><em>cox VI b</em></td>
<td>0.94 ± 0.13</td>
<td>1.01 ± 0.07</td>
<td>0.127</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cox VI c</em></td>
<td>1.06 ± 0.07</td>
<td>1.11 ± 0.13</td>
<td>0.247</td>
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<td></td>
</tr>
<tr>
<td><strong>Glucose Transport and Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lvglut 1</em></td>
<td>0.89 ± 0.08</td>
<td>0.93 ± 0.09</td>
<td>0.563</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lvglut 2</em></td>
<td>0.83 ± 0.21</td>
<td>0.93 ± 0.37</td>
<td>0.442</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ldh</em></td>
<td>0.91 ± 0.29</td>
<td>1.03 ± 0.44</td>
<td>0.798</td>
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</tr>
<tr>
<td><em>pk</em></td>
<td>0.95 ± 0.11</td>
<td>0.99 ± 0.19</td>
<td>0.752</td>
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<tr>
<td><em>hk</em></td>
<td>0.89 ± 0.17</td>
<td>1.03 ± 0.20</td>
<td>0.160</td>
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<tr>
<td><em>fbp</em></td>
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<td>0.574</td>
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<tr>
<td><em>pepck</em></td>
<td>1.14 ± 0.69</td>
<td>0.88 ± 0.32</td>
<td>0.721</td>
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</tr>
</tbody>
</table>
Table 5: The long-term effect of the early stimulus on the mRNA levels (normalized by the reference gene ef1a) of the genes coding digestive, intermediary and energy metabolic enzymes or transporters measured at the whole body level of *L. vannamei* at the end of experiment; juvenile (PL 29). Data were presented as mean ± SD (n=6 whole body per experimental group). CTL: control group (no feed restricted group). RES: restricted group (feed restricted group). After confirming that these data were not normally distributed assessed by Shapiro-Wilk test, statistical differences were evaluated by Wilcoxon test; differences are statistically significant when *P*<0.05.

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>CTL</th>
<th>RES</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Means ± SD</td>
<td>Means ± SD</td>
<td></td>
</tr>
<tr>
<td><strong>Digestion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lipase</td>
<td>1.23 ± 0.45</td>
<td>0.94 ± 0.39</td>
<td>0.234</td>
</tr>
<tr>
<td>preamylase</td>
<td>1.28 ± 0.28</td>
<td>1.07 ± 0.36</td>
<td>0.234</td>
</tr>
<tr>
<td>chymotrypsin</td>
<td>1.02 ± 0.18</td>
<td>1.10 ± 0.62</td>
<td>0.878</td>
</tr>
<tr>
<td>trypsin</td>
<td>1.17 ± 0.64</td>
<td>1.05 ± 0.56</td>
<td>0.878</td>
</tr>
<tr>
<td><strong>Lipid Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fas</td>
<td>1.14 ± 0.29</td>
<td>1.05 ± 0.18</td>
<td>0.442</td>
</tr>
<tr>
<td><strong>Amino Acid Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gs</td>
<td>1.24 ± 0.35</td>
<td>1.09 ± 0.25</td>
<td>0.574</td>
</tr>
<tr>
<td>gdh</td>
<td>1.02 ± 0.15</td>
<td>1.18 ± 0.23</td>
<td>0.105</td>
</tr>
<tr>
<td><strong>Energy Metabolism</strong></td>
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<td></td>
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<td>1.04 ± 0.10</td>
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<tr>
<td>cox VI a</td>
<td>1.16 ± 0.18</td>
<td>1.24 ± 0.28</td>
<td>0.574</td>
</tr>
<tr>
<td>cox VI b</td>
<td>1.16 ± 0.07</td>
<td>1.09 ± 0.23</td>
<td>0.721</td>
</tr>
<tr>
<td>cox VI c</td>
<td>1.07 ± 0.08</td>
<td>1.25 ± 0.25</td>
<td>0.105</td>
</tr>
<tr>
<td><strong>Glucose Transport and Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lvglut 1</td>
<td>1.07 ± 0.20</td>
<td>1.14 ± 0.02</td>
<td>0.505</td>
</tr>
<tr>
<td>lvglut 2</td>
<td>1.25 ± 0.41</td>
<td>0.96 ± 0.34</td>
<td>0.234</td>
</tr>
<tr>
<td>ldh</td>
<td>0.90 ± 0.12</td>
<td>1.36 ± 0.44</td>
<td>0.038</td>
</tr>
<tr>
<td>pk</td>
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<td>1.51 ± 0.38</td>
<td>0.015</td>
</tr>
<tr>
<td>hk</td>
<td>1.28 ± 0.25</td>
<td>1.06 ± 0.19</td>
<td>0.049</td>
</tr>
<tr>
<td>fbp</td>
<td>1.15 ± 0.16</td>
<td>1.19 ± 0.15</td>
<td>0.955</td>
</tr>
<tr>
<td>pepck</td>
<td>1.12 ± 9.16</td>
<td>0.98 ± 0.30</td>
<td>0.574</td>
</tr>
</tbody>
</table>
Figure 1.
Highlights

- Moderate feeding restriction (40% less feeding) stimulus during 4 days of the protozoea phase was possible without any deleterious effect on the shrimp performance and survival.
- Early nutritional stimulus in protozoea shrimp was able to induce at long term slight modifications of the shrimp metabolism at a molecular level.
- Early nutritional stimulus in protozoea shrimp did not modify at long term the growth performance.

Statement of Relevance

The present study had the objective to test the feeding restriction stimulus at the protozoea stage for whiteleg shrimp as a factor of the nutritional programming. We submitted two experimental groups, normal feeding protocol (CTL) and feed-restricted group with 40% less feeding rate (RES) during 3 days. This was followed by a common rearing period. The mRNA level of *preamylase* and *lvglut 2* were upregulated as a direct effect of the feed restriction. As the long-term effect of stimulus, no modifications were observed on the biomarkers related to the digestion. Nevertheless, we observed some genes involved on the energy and glucose metabolism, which had altered mRNA levels in relation to the early stimulus. Overall, even though we did not observe any negative effects of the early stimulus on the growth performance and survival of the shrimp, this stimulus did not seem to be efficient to induce important effects on the metabolism after 40 rearing days.

Acknowledgments

We acknowledge ANRT and CNPq for funding Luis Paulo Araujo Lage (CIFRE-Brasil/CNPq - GDE – France / Number 217420/2014-1). All the experiments were funded by Neovia NSA and Labomar’s marine aquaculture experimental station. We thank LABOMAR staff for the daily support. We thank Daniel Arana Braidi for the help with hatchery and nursery informations. We thank CELM Aquicultura S/A for the shrimp larvae and microalgae (Hudson Makson Rocha Lucena).
Chapter 5

General discussion
Figure 5.1-1. Targeted window (for the programming) of the protozoa: (Z1-Z2-Z3) of the early development of *L. vannamei*, characterized by high molecular plasticity (Adapted from the publication 1). gs; glutamine synthase.
5.1 The choice of two developmental windows (protozoea and post-larvae) to perform the nutritional programming in whiteleg shrimp

The concept of nutritional programming is well known in mammals and more recently, several studies have been accomplished on fish species (Duque-Guimarães and Ozanne, 2012; Balasubramanian et al., 2016; Panserat et al. 2017; Rocha 2016; Turkmen et al. 2017). However, to our knowledge, no studies about programming were performed in shrimp prior to this Ph.D thesis. Thus, this thesis aimed at studying the nutritional programming approach in the whiteleg shrimp *Litopenaeus vannamei*, using the early feeding restriction as a trigger to persistent molecular modifications and better utilization of alternative diets. For this purpose, we need to, first, investigate which are the sensitive windows of the early development as potential stages to perform the nutritional stimulus (cf publication 1). The main hypothesis is that the time when gene expression is modified (increase/decrease) is the time of molecular plasticity and so becomes a good candidate period for programming animals. With a lack of information about the molecular ontogenesis of actors involved in whiteleg shrimp, we studied the ontogenesis of gene expressions involved in the digestion, intermediary and transport metabolism, during the embryo and larval development. Mainly, two developmental windows were considered as candidates to perform environmental stimuli for the nutritional programming.

(i) The first-feeding protozoea stage, is associated with high molecular plasticity for the digestive enzymes (preamylase, triacylglycerol lipase and trypsin), as previously observed by Wei et al. (2014). Among other genes, which are also induced for their expression at the protozoea phase, are those related to the amino acids, energy and glucose metabolism. Figure 5.1-1 highlights the results. Indeed, the high molecular plasticity of the protozoea phase is probably linked to the onset of exogenous feeding habits for the shrimp, similar to the “first feeding” stage in fish (Geurden et al., 2014; Rocha et al., 2014) as reported by Mennigen et al. (2013) which have a high level of plasticity of metabolism at the molecular level. However, the protozoea animals are considered to be fragile; protozoea stage could be a difficult phase to manipulate and any severe nutritional manipulation could block moulting which is paramount to achieve success in shrimp hatchery. For example, the protozoea-2 syndrome, is characterized by a reduction of feeding rate during the late sub-stages of protozoea Z1 and early Z2, impairing the metamorphosis with high rates of mortalities, caused by bacterial
infection, Vibrio harveyi in Latin America (Morales-Covarrubias, 2008) and Vibrio alginolyticus in India (Kumar et al. 2017).

(ii) Meanwhile, the second developmental window of shrimp, i.e., the early post-larvae stage, was associated with high plasticity of the gene expressions in particular (but not only) related with the glucose metabolism (Figure 5.1-2). Contrary with the early protozoea stage, the post-larvae stage corresponds to animals which are more resilient to environmental changes. At this stage, animals can be fed with inert food and are less dependent of the live food (microalgae and/or nauplii Artemia). Thus, it is the preferential window to test the nutritional programming, mainly through nutritional stimuli corresponding to the feed restriction.

Crustaceans, during the early development goes through a typical metamorphosis process (embryo, nauplii, protozoea, mysis and, post-larvae steps) before becoming an adult. Both morphology and physiology features change dramatically over the developmental periods, which are accompanied by different behaviors, habitats, feeding strategies and metabolism (Dall et al., 1990; Fang and Lee, 1992 Jones et al., 1992; Le Moullac et al., 2002). Metamorphosis can be characterized as the period in the life cycle of an animal in which an immature individual undergoes drastic anatomical and physiological changes to develop into an adult (Medina, 2009). Thus, the drastic modification on the metabolism, morphology and physiology features over the development of shrimp, strongly suggested that the choice of the sensitive windows was mandatory for the progress of our study.

On the other hand, in mammals and other viviparous animals (new born animals which are fully or almost developed), the embryo development occurs within the parental body involved by placenta to furnish the nutrients necessary for development and remove the excreta. The time frame for which the programming can occur is often confined to the sensitive periods of the early life, such as during the intra-uterine (via maternal nutrition) or the neonatal nutrition. Those environmental modifications, such as nutritional variations, at the early life are often correlated with non-communicable disease (i.e., diabetes, obesity and cancer) on the adulthood life (Lillycrop and Burdge, 2012; Patel and Srinivasan, 2002; Rando and Simmons, 2015). The effects of nutritional programming are likely to be more efficient when stimulus is applied on poorly differentiated cells or tissues, when the organism through the process of developmental plasticity will be sensitive to environmental perturbations (Gluckman et al.
Figure 5.1-2. Targeted window (for the programming) of the post-larvae stage 1 PL1 of the early development of *L. vannamei* (Adapted from the publication 1). *pepck*: phosphoenolpyruvate carboxykinase; *lvglut1*: *L. vannamei* glucose transporter 1.
2005). Furthermore, the period of plasticity for which the environmental cues can exert persistent nutritional programming in adults can be extended up to the early postnatal life, period in which several regulatory mechanisms continue their development (Patel et al., 2009; Srinivasan and Patel, 2008). In conclusion, similarly to the prenatal period of mammals, the embryo/larval development of crustaceans has a high molecular plasticity suitable to test the programming concept.

In conclusion we had selected two developmental windows, i.e., protozoea and post-larvae stages for the stimuli based on the molecular plasticity of the gene expressions for metabolism and digestion.
Figure 5.2-1. Different levels of feeding restriction at the early development of *L. vannamei*: 70% at the post-larvae stage and 40% at the protozoea stage.

Figure 5.2-2. Summary of the direct effect on the early feed restriction at two sensitive windows (protozoea 40% and post-larvae 70%) on the metabolism.
5.2 The early feed restriction stimulus was performed at two developmental stages in whiteleg shrimp

In this thesis, we decided to use the early feed restriction as an environmental stimulus to test for the first time in the whiteleg shrimp the concept of nutritional programming. In mammals, it is well known that nutritional manipulation applied on the sensitive stage of pre or neo-natal period may have long-term effects on metabolism or physiology (Barker and Osmond, 1986; Burdge and Lillycrop, 2010; Lucas, 1998; Metges et al., 2014; Patel et al., 2009; Spencer, 2012).

According with Armitage and colleagues (2004), several studies have been accomplished using different species, different feeding regimes and diets in order to evaluate the early origin of the development of the metabolic syndrome in adults. The caloric restriction (through restriction of feeding) was classified as mild, moderate and severe (30, 50 and 70% of caloric restriction, respectively), based on this data we performed two feed restrictions in shrimp (Figure 5.2-1): i) severe (70%) and ii) moderate (40%). These restrictions were characterized by a reduction of feed allowance (dried feed and microalgae) on the ratio related to each stimulus. Albeit, the allowance of all the nutrients were restricted, but crude protein (CP) intake was severely reduced because it accounted for the major macronutrient in the formula according to the feed manufacturer (BernAqua NV, Olen, Belgium) (Tables 3.3.2; 3.4.2).

The early stimuli at both protozoa and post-larvae stages were efficient to test the programming because no differences in survival and growth performance were observed in both cases and so no speculation on genetic selection could be raised (cf publications 2 and 3). However, direct effects of the restriction on the metabolic gene expressions were relatively low (Figure 5.2-2), except for the trypsin gene which was down-regulated in the restricted group (publication n°2).

It is not surprising to have only this gene (involved in protein digestion) to be sensitive to early stimulus as the main factor changing during the stimuli is the level of dietary proteins. Indeed, it is reported that L. vannamei can adapt digestive enzyme activities, including trypsin, in response to the dietary crude protein levels (Carrillo-Farnés et al., 2007; Lee et al., 1984; Puello-Cruz et al., 2002; Wei et al., 2014a). By contrast, the mRNA levels of premylase and glucose transporter 2 (lvglut 2) were higher on the feed-restricted group at the protozoa stage (publication 3). These differences between the two experiments of feed restriction could be due either to the window of the development or to the strength of the stimulus. In conclusion, all these findings at the protozoa and post-larvae stages suggest the early restriction stimulus is not strong (long) enough to induce changes at a molecular level for metabolic genes.
In summary, we used as a stimulus the feed restriction strategy (decrease of feed intake, mainly proteins and energy) for the protozoea and post-larvae stages. The absence of a decrease in survival between experimental groups leads us to conclude these stimuli could be used for testing the nutritional programming concept. However, the direct effects of the stimuli were low for all the metabolic gene expressions, suggesting that it could be possible to test stronger restriction stimuli (stronger level of feed restriction, longer duration of the stimulus) in the future.
Figure 5.3-1. Causes and consequences of the early nutritional stimulus on the nutritional programming and development of the non-communicable diets on mammals (adapted from Duque-Guimarães and Ozanne, 2013).
5.3 Was it possible to program the whiteleg shrimp through an early nutritional stimulus?

Studies on mammals have shown that severe caloric restriction during the pregnancy period was associated with descendant mitochondrial abnormalities that consequently decreased the ATP productions levels (Mayeur et al. 2013). Furthermore, other studies that used the macronutrient restriction approach, reducing the level of dietary protein to 8% protein with increased levels of CBH, in order to maintain an isocaloric diet, through the pregnancy period, revealed that the offspring of feed-restricted mothers were born with a low weight, followed by induced an insulin resistance in adults (Petry et al., 2001; Fernandez-Twinn et al., 2005). The main consequences of the nutritional stimulus on the metabolic programming are schematized in Figure 5.3-1. It is clear that an early caloric restriction can be related to some changes in intermediary metabolism in adult shrimp; this is why we decided to test the early restriction stimulus and its potential long-term effect on the metabolism of the adult during this thesis.

5.3.1 It was possible to program *L. vannamei* juveniles after an early feed stimulus (feed restriction) at the post-larvae stage.

An important finding of this thesis was the demonstration for the first time that it was possible through nutritional stimulus at the early stage of post-larvae PL1 to “program” *L. vannamei*. This programming results in better growth performance and persistent modifications for digestive and metabolic actors at the molecular level (cf publication 2). The early 70% feed restriction stimulus at the post-larvae stage is a suitable tool (*i.e.*, suitable nutritional strategy) for the nutritional programming in shrimp. This stimulus applied did not affect the survival of the animals evaluated at the end of hatchery phase, thus suggesting no genetic selection (as explained in the previous chapter). Furthermore, this kind of stimulus does not demand a high level technology to carry out and, so it could be easily applied in commercial hatcheries.

Although this stimulus did not affect the metabolism at short-term (just after the stimulus, 4th day; cf previous chapter) effects of the nutritional history were observed after the dietary challenge phase, *i.e.*, the so-called the “long-term” or “programming” effect.

During this thesis, we can highlight that the early feed-restriction at post-larvae stage had a long-term impact on: (i) the growth performance; (ii) the digestive enzymes and (iii) the metabolic enzymes in the hepatopancreas. Regarding the growth performance, the severe early feed restriction stimulus improved shrimp growth performance after the dietary
Figure 5.3.1-1. Summary of the data obtained in the thesis about the nutritional programming on *L. vannamei* linked to the 70% feed restriction stimulus at the early post-larvae stage. FBW: final body weight; SGR: specific growth rate; *gs*: glutamine synthetase; *cox VI b*: mitochondrial cytochrome C oxidase subunit VIb; *lgvlut 1* and *2*: glucose transporter 1 and 2; *pk*: pyruvate kinase (adapted from publication n°2).
challenge with the different diets. The final body weight was strongly affected by the early stimulus, such as for the P35 (supplementary data) and P43 diets. While no difference was observed on the final body weight for the CTL group, feed-restricted animals improved their final body in response to an increase in the dietary protein content. Regarding the digestion and metabolism, the expression of the enzymes was also modified depending of the early stimulus, showing without ambiguities, the existence of programming at a molecular level in shrimp. Finally, the mRNA levels of lipid, preamylase and trypsin, were affected by the nutritional history after the dietary challenge, resulting on a down regulation of all these genes for the feed-restricted group. The same pattern of down-regulation was observed to the genes that code for enzymes related to the glucose (lvglut 1, 2 and pk) and energy metabolism (cox VI b). These data cannot clarify some hypothesis to explain the improved growth performances that we observed with the early-restricted group.

As with shrimp, studies performed in mammals have shown that undernutrition during the early life (prenatal and/or lactation) demonstrate a relationship with the development of metabolic variations, in particular in relation to metabolic diseases, i.e., development of diabetes, hypertension and obesity (Burdge and Lillycrop, 2010; Jiménez-Chillarón et al., 2012, Lillycrop and Burdge, 2012). Finally, it is reported in mammals that early (pre and postnatal period) malnutrition exerts modification on the lipid, glucose and energetic metabolism (Vickers et al., 2003; Devaskar and Thamotharan, 2007; Thompson et al., 2007) as we have observed in shrimp (Figure 5.3.1-1). Thus, new feeding strategies can be implemented on the shrimp production linked to the nutritional programming concept. Nevertheless, further studies should be performed to better understand the mechanisms at the origin of these permanent molecular modifications, such as investigations on epigenetic marks. Although the DNA methylation was evaluated on this study showing no significant difference between groups (publication n°2, complementary data), we must be careful with these preliminary data to avoid any over-interpretation about the absence of epigenetic variations as more analysis of DNA methylation are required using other more sensitive approaches. Research of variations in other epigenetic marks (such as those on the histones) could also be interesting.
Figure 5.3.2-1. Summary of the data obtained in the thesis about the nutritional programming on *L. vannamei* linked to the 40% feed restriction stimulus at the early protozoea sub-stage Z1 sub-stage. *atpase a* and *b*: mitochondrial ATP synthase subunit alpha and beta; *ldh*: lactate dehydrogenase; *pk*: pyruvate kinase and *hk*: hexokinase (adapted from publication n°3).
5.3.2 A moderate feed restriction stimulus at early protozoea stage did not strongly modify in the long-term the growth performance, digestion and metabolism of *L. vannamei*.

During this Ph.D., we evaluated the effect of the early stimulus at the protozoea sensitive developmental window, through a specific feeding restriction protocol (40% lower than normal feed intake) (cf publication n°3). At the end of experimental period (40th day), no differences in long term effects were observed in shrimp growth performance. Additionnaly, the moderate early stimulus did not exert a lot of persistent modifications of the metabolism at the molecular level. Indeed, the major modifications were observed for some genes in relation to the energy metabolism (*atpase a and b*) and glucose metabolism (*ldh*, *pk* and *hk*), which were up-regulated on the feed-restricted group, except for the hexokinase (Figure 5.3.2-1). It is important to conclude here the same type of stimuli applied at different stages of development (publication n° 2), have exerted different modifications of biomarkers related to the glucose and energy metabolism in *L. vannamei*. We can also suspect that the level of feed restriction (40% in the present study versus 70%) was not sufficient to induce major long-term changes at in shrimp. Moreover, it is difficult to compare the two experiments of programming because this experiment was concluded before the first one. Indeed, a longer experiment must be performed in the future in order to test, as for the post-larvae experiment, the use of different diets in juvenile shrimp.

In conclusion, the nutritional programming of shrimp was demonstrated after an early feed restriction stimulus (70%) at the post-larvae stage, showing an improvement of the growth performance and some permanent changes of digestive/metabolic gene expression in juvenile shrimp. However, the stimulus applied at the protozoea stage was not effective for programming the growth performance. This may be linked to the level of the restriction which was not high (40%) in order to avoid any mortalities in the fragile protozoea animals.
Table 5.4. Formula cost of diets used in the challenge phase and the revenue. Values were estimated considering the exchange rate of $1.00 USD = R$ 3.08 BRL and Brazilian market shrimp price R$ 29.00 BRL/kg; 9.0 g body weight mean, on 17th February 2018.

<table>
<thead>
<tr>
<th></th>
<th>P30</th>
<th>P37</th>
<th>P43</th>
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<tr>
<td>Cost per ton (USD)</td>
<td>672.8</td>
<td>702.8</td>
<td>765.0</td>
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<tr>
<td>Savings relative to control (%)</td>
<td>13.7</td>
<td>8.9</td>
<td>-</td>
</tr>
<tr>
<td>Revenue (USD)</td>
<td></td>
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<tr>
<td>CTL</td>
<td>16.65</td>
<td>16.49</td>
<td>17.58</td>
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<tr>
<td>RES</td>
<td>17.82</td>
<td>16.69</td>
<td>17.92</td>
</tr>
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5.4 The decrease of the dietary proteins/carbohydrates ratios in juveniles of whiteleg shrimp: consequences on growth performances and molecular hepatic metabolism.

To maintain aquaculture as a sustainable activity, there is a consensus to reduce fishmeal use in aquafeeds (Hardy, 2010; Klinger and Naylor, 2012 Naylor et al., 2009). As opposed to protein, aquatic animals do not have a dietary nutritional requirement for carbohydrates (CBH). Shrimp are also able to cope with CBH-free diets because they synthetize glucose from non-glucose substrate by the gluconeogenesis pathway (NCR, 2011). However, the dietary inclusion of CBH from 20 up to 30% is generally well accepted (Wang et al., 2016); indeed, the use of CBH, the cheapest formula ingredient, as source of energy has a sparing effect on proteins (Cho and Kaushik 1990). Several enzymes of the CBH digestion and glucose metabolism have been detected on *L. vannamei* (NRC, 2011; Wang et al., 2016; Wei et al., 2014b). Moreover, they are able to adapt their digestive enzymes activity in response to the diet profile and ingredient composition (Carrillo-Farnés et al., 2007; Cuzon et al., 2004; Gaxiola et al., 2005; Li et al., 2011; Puello-Cruz et al., 2002; Rosas et al., 2000), suggesting the species capable to utilizing different ingredient sources.

Thus, regardless of the nutritional history parameters, one objective of this thesis was also to evaluate the effects of fishmeal replacement by plant ingredients, which are naturally rich on CBH and consequently, modified the dietary proteins/CBH ratios (publication 2). It is important to note that diets used in this study have been formulated to have a practical interest; indeed, feed formulations were done by the Neovia company in order to dilute the dietary protein content (and the dietary inclusion of fishmeal) by increasing CBH level. Thus, fishmeal was replaced by alternative and practical ingredients (soybean meal, wheat flour and cassava), supplemented with DL-Methionine and L-Lysine to account for a lower amino acids composition from these ingredients. Of course, and different from purified diets, diets were composed of many different raw ingredients which could have associated with their digestibility, the bioavailability of their nutrients, and the presence of anti-nutritional factors (ANF). Despite these limitations, it was possible to analyze the effect of the different dietary proteins/CBH ratios on the growth performance and metabolism of the juvenile shrimp. Considering that feed cost can represent more than half of the production costs in shrimp aquaculture, the development of new diet formulations is essential. Indeed, for diets used in the dietary challenge in publication n°2, we extrapolated the revenue for the different diets (Table 5.4) based on: local real market prices, exchange rate and yield per treatment, relative to the period that the experiment was carried out, which were R$ 29.00 BRL/kg the real price.
**Figure 5.4-1.** Summary of the effect of diet challenge on the growth performance and gene expression of RNA extracted from hepatopancreas. CBH: carbohydrate; CP: crude protein FBW: final body weight; FCR: feed conversion ratio; *ldh*: lactate dehydrogenase and *pepck*: phosphoenolpyruvate carboxylase.
market (related to shrimp with 9.0 g body weight mean) and a exchange rate on $ 1.00 USD = R$ 3.08 BRL.

After a 70-day challenge, shrimp fed diets with the lowest protein/CBH ratio had a lower final body weight compared to other. Albeit we did not observe any difference on the apparent feed intake (g of feed for stoked/shrimp), FCR increased when the level of dietary proteins decreased. In general, these findings were expected as proteins play an important role in the aquafeed formulation, being the major limiting nutrient for growth. The dietary protein requirement for *L. vannamei* during the juvenile phase ranges from 30 up to 36% on a digestive basis (Colvin and Brand, 1977; Kureshy and Davis, 2002; NRC, 2011; Smith *et al.*, 1985). Thus, all the formulas theoretically met the requirement on crude protein. However, it is important to conclude that the value on digestible protein were lower than the required for the species which could impact the growth performance, as we observed on the diet P30.

On this study we analyzed the effects of the dietary challenge on the hepatopancretic mRNA level of several biomarkers. A decrease of the dietary protein/CBH ratio have increased the gene expression of the enzyme coding for CBH digestion (*preamylase*). This result is surprising (lower level of amylase gene expression with higher content of CBH in diet) and cannot be clearly explained. In regards to the genes of the metabolism (amino acids and glucose metabolisms), a regulation of the expression in relation to the dietary proteins/CBH ratios was observed. The mRNA levels of glutamate dehydrogenase (**gdh**) were lower in shrimp fed with the low protein/CBH diet, suggesting the low dietary protein content was associated with a decrease of the amino acids catabolic capacity. As expected, the capacity to *de novo* synthesis of glucose through two gluconeogenic enzymes (**fbp** and **pepck**) was reduced in shrimp fed with the high dietary carbohydrate content (Figure 5.4-1).

Based on these data, we can conclude that *L. vannamei* can adapt to the new diets with different proteins/CBH ratios. From a practical point of view, the successful reduction of fishmeal in aquafeed formulation is highly important for the reduction of feed cost and the preservation of marine resources.

In conclusion, both amino acids and glucose metabolism were regulated by the dietary proteins/CBH ratios. Thus, *L. vannamei* is able to adapt its metabolism in relation to the diet composition. Indeed, the use of plant-based diets is feasible to produce shrimp. This accost savings in formulation. However, the low protein/CBH ratio decreased the FBW of shrimp as well their gained yield. Thus, the reduction of the dietary proteins/CBH ratio in practical diets can limit shrimp growth performance.
Figure 5.5-1. Summary of the interactions between the nutritional history and the diets on the final body weight FBM (g) of *L. vannamei*; COX VI c: mitochondrial cytochrome C oxidase subunit VI c; LV GLUT 1: glucose transporter 1 and Nutritional history (CTL: control; black box and RES feed-restricted group; white box). P43: 43.3% CP and 29.3% CBH; P37: 36.9% CP and 37.6% CBH and P30: 29.5% CP and 45.5% CBH.
5.5 Could the use of the different diets be modified by the early feed restriction stimulus?

After the dietary challenge period, we did not observe a lot of interaction between the nutritional history and the dietary challenge (cf publication n°2 (Figure 5.5-1)). It is difficult to discuss the global significance of the interactions detected. Concerning the growth performance, interaction was found for only the FBW, which increased with the increased proteins/CBH ratio only significantly in the restricted group. Related to gene expression of the genes extracted from shrimp hepatopancreas, only the mRNA levels of the energy metabolism cox VI c and the glucose transport ivglut1, were subjected to interactions. However, discussing the results imposes a lot of challenges.

As conclusion few interactions were observed for the dietary challenge and the programming (stimulus). However, modification of shrimp growth performance in response to the diets seemed to be higher in the feed-restricted group, suggesting that the nutritional programming can improve the nutrient use.
Chapter 6

Conclusions and perspectives
Conclusions

In recent years, several studies have been performed in vertebrates (fish and mammals) using the approach of nutritional programming (Clarkson et al., 2017; Izquierdo et al., 2015; Liu et al., 2017; Panserat et al., 2017; Vickers, 2014). In general, data show that adverse early events, such as environmental conditions and pre and neo-natal, can trigger long-term modifications leading to differences of metabolism and/or phenotype.

Overall, the studies conducted within this thesis showed for the first time that it is possible to achieve a nutritional programming in a crustacean, *Litopenaeus vannamei*, through early nutritional stimuli as observed in mammals.

To summarize, the following conclusion can be highlighted:

- The early developmental larval stages (protozoea and early post-larvae) of the whiteleg shrimp have a high molecular plasticity, indicating the existence of optimal windows to assess the nutritional programming. Albeit the post-larvae stage seems as a late developmental window, animals are more resistant and the strength of stimulus (feed restriction) can be more severe.

- The nutritional stimulus (feed restriction) used in this thesis was suitable to perform the nutritional programming for shrimp, because it did not impact survival, preventing any genetic selection of the animals (which is not compatible with the programming concept). In addition, feed restriction is a simple tool for farmers because it does not require high technology to be performed. It can be easily applied in commercial hatcheries.

- The direct effects of the early stimulus were not strong at the molecular level for metabolism and digestion. The programming after the post-larvae stage stimulus was associated with increased growth performance and changes of the expression of some of the metabolic genes after dietary challenge of juveniles, suggesting this nutritional strategy (the programming concept) could be adequate for improving shrimp nutrition in the future.

- The developmental window of protozoea had high plasticity at the molecular level. However, the effect of the stimulus (by moderate feed restriction) did not exert mid-long phenotype modification (growth performance), despite some adaptation at the molecular level for glucose and energy metabolism. Thus, the potential of this developmental window for nutritional programming in furthermore questionable.
Figure 6.1.1.1. Summary of the possible future research areas for programming shrimp.
Perspectives

The following chapter describes some of the research areas which could be developed in the future for testing and improving the programming approach in shrimp.

6.1.1.1 Further research for new stimuli

We tested in this thesis feed restriction as a stimulus for programming shrimp. Even though this stimulus seems to be effective at modifying the long-term metabolism and use of diets, other stimuli could be tested (Figure 6.1.1.1):

We can propose new nutritional stimuli in shrimp, such as those linked to the diet composition and nutrient profile (high levels of carbohydrates, vitamins, minerals, etc). Indeed, we know that in vertebrates the dietary composition can have a strong influence, through programming, on the fate of the adults (Duque-Guimarães and Ozanne, 2013; Langley-Evans, 2009).

Environmental stimuli, which are not nutritional, can influence the metabolism at long-term. For example, temperature and salinity of the rearing water are potential modifiers of metabolism in fish (Burgerhout et al., 2017; Liu et al. 2017; Mazurais et al. 2014; Pittman et al., 2013). Therefore, they can act as good candidates for new stimuli to test the metabolic programming in shrimp. Moreover, these stimuli could be used at very early stages of the embryogenesis, which can be promising for programming.

The stimuli we used in the present Ph.D. work were relatively short in time (3 days or 4 days). We can hypothesize that a longer stimulus period (time frame) or a stronger one (starvation) could be more efficient for programming. However, we must be careful with the stimulus to avoid shrimp mortality and genetic selection. Furthermore, it is well known that high shrimp stocking density is a stressful condition and can trigger diseases, leading to a loss in growth performance and poor survival (Araneda et al., 2008; Sookying et al., 2011). Thus, in addition to a stronger stimulus, the combination of the challenge (diet) period with a high stocking density, could promote larger differences in phenotype and/or metabolism.
The final conclusion of this thesis is that the nutritional programming could play an important role to alter the metabolic utilization of several nutrients, enabling the development of new feeding strategies to improve the use of plant-based diets (alternative diets) for shrimp production in the future.

6.1.1.2 Evaluation of new developmental windows to perform the stimuli

In this thesis we selected two developmental windows (protozoea and post-larvae stages) to carry out the early stimuli for programming the adults. The hypothesis was that we chose the window of development which corresponds to the stronger modifications of gene expression (mainly the induction of gene expression for metabolic genes). We can even speculate that we did not choose the most sensitive developmental window: the “golden” developmental windows could be just before these periods in order to modify the gene expression permanently. This needs to be tested in the future.

We also suspect that parental nutrition (broodstock nutrition) of shrimp could be interesting to modify the fate of the offsprings (through epigenetic modifications of the gametes, for example) as proved recently in animals (Izquierdo et al., 2015; Turkmen et al., 2017).

6.1.1.3 Research of new biological targets in order to test the programming concept

During this thesis, we chose to test the programming concept to analyze some candidate genes in the domain of intermediary metabolism. Our analysis shows that some of the genes are expressed at different levels linked to the programming which is important as the concept of programming is mainly linked to molecular mechanisms (Best et al., 2018, Duque-Guimarães and Ozanne, 2013; Gavery and Roberts, 2017). However, to be sure these changes of gene expression could be associated with regulation of the intermediary metabolism, other analyses must be performed. Analysis of enzyme activities, protein level, and metabolic fluxes would be very important to conclude on the long-term impacts of the metabolism programming.

On the other hand, use of not a priori approaches based on the “omics” technologies (transcriptomics, proteomics, metabolomics) could also be useful (even though complex) for researching new pathways which could be influenced by the programming.

The last but not the least, it is evident that analysis of epigenetics marks in the animals tested for programming must be done in the future. These data must be analyzed not only - as we did with a low sensitive approach about DNA methylation - but using new technologies. Moreover, other epigenetics marks associated with histone modifications, miRNAs could be also very interesting, opening new perspectives on the understanding of the mechanism at the origin of the nutritional programming in shrimp.
Bibliography


Augustin, R., 2010. The protein family of glucose transport facilitators: it’s not only about glucose after all. IUBMB Life 62, 315e333.


Colvin, L.B., Brand, C.W., 1997. The protein requirement of penaeid shrimp at various life cycle


par des facteurs hétérologues et homologues. Université de Bretagne Occidentale, Brest, France.


Okano, M., Bell, D. W., Haber, D. A., Li, E., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247–257.


177


the diet of Cirrhinus mrigala (Hamilton, 1822) fry. Aquaculture 258, 680–684. doi:10.1016/j.aquaculture.2006.03.049


Torrens, C., Brawley, L., Anthony, F.W., Dance, C.S., Dunn, R., Jackson, A.A., Poston, L., Hanson, M.A., 2006. Folate Supplementation During Pregnancy Improves Offspring Cardiovascular Dysfunction Induced by Protein Restriction. Hypertension. 47. doi:10.1161/01.HYP.0000215580.43711.d1


192.


List of publications
Publication during PhD:


(3) Lage, L.P.A., Weissman, D., Serusier, M., Putrino, S.M., Baron, F., Guyonvarch, A., Tournat, M., Nunes, A.J.P., Panserat, S. The 4 days feeding restriction at the protozoa stage had a little effect at long term on metabolic gene expressions in whiteleg shrimp (*Litopenaeus vannamei*). (To be submitted).

Communications


Résumé: Programmation nutritionnelle de la crevette du Pacifique à pattes blanches *Litopaneus vannamei*

Ce travail de thèse a eu pour objectif de tester pour la première fois le concept de programmation nutritionnelle chez la crevette *Litopaneus vannamei*. La première question était de savoir à quel moment le stimulus précoce devait se faire lors du développement de la crevette. Dans ce but, nous avons déterminé deux fenêtres de développement (stades protozoea et post-larvae) pendant lesquelles la plasticité moléculaire pour le métabolisme semblait optimale (*publication n°1*). La deuxième question portait sur le choix du stimulus environnemental que nous voulions utiliser. Nous avons pris comme stimulus nutritionnel la restriction de la prise alimentaire (restriction énergétique) sachant que ce stimulus pouvait avoir des impacts forts sur le métabolisme de l’adulte chez de nombreuses espèces animales. Nous avons pu démontrer qu’une restriction alimentaire de 40 % (40% de baisse de quantité d’aliment distribuée) dans la phase protozoe (4 jours) et de 70% dans la phase post-larvae (3 jours) était réalisable sans que cela n’induisse de baisse de survie et de pertes de performances de croissance des animaux. La restriction alimentaire au stade protozoe n’a pas permis d’observer à long terme des modifications des performances de croissance et du métabolisme (au niveau moléculaire) (*publication n°3*). Par contre, la restriction alimentaire au stade post-larvae a été un succès concernant la programmation : les performances de croissance, l’utilisation des aliments (avec différents ratios de protéines/glucides) et le métabolisme (au niveau moléculaire) ont été (positivement) affectés par le stimulus précoce chez les animaux juvéniles (*publication n°2*). Nos travaux originaux et prometteurs nous permettent donc d’envisager dans le futur des expériences de programmation précoce (via la nutrition ou autres facteurs environnementaux) afin de piloter la nutrition des crevettes en aquaculture.

**Mots clés**: crevettes, aquaculture, programmation nutritionnelle, nutrition

Abstract: Nutritional programming of Pacific whiteleg shrimp *Litopaneus vannamei*

This thesis work aimed to test for the first time the concept of nutritional programming in *Litopaneus vannamei* shrimp. The first question was when early stimulus should be performed during shrimp development. For this purpose, we determined two developmental windows (protozoea and post-larvae stages) during which the molecular plasticity for the metabolism seemed optimal (*publication no. 1*). The second question was about choosing the environmental stimulus we wanted to use. We took as nutritional stimulus the feed restriction (energy restriction), reported that this stimulus promotes strong impacts on the metabolism in the adulthood of many species of mammals. We were able to demonstrate that a dietary restriction of 40% (40% reduction in quantity feed allowance than normal) at the protozoea phase (4 days) and 70% in the post-larvae phase (3 days) was suitable without deleterious impacts on survival and growth performance of the animals. The feed restriction at protozoea stage did not show long-term changes in growth performance and metabolism (at the molecular level) (*publication no. 3*). In contrast, post-larvae the feed restriction was successful for the programming: growth performance, food utilization (with different protein/carbohydrate ratios) and metabolism (at the molecular level) were (positively) affected by the early stimulus during the development (*publication no. 2*). Our original and promising work allows us to envisage in the future early programming experiments (via nutrition or other environmental factors) to pilot shrimp nutrition in aquaculture.

**Keywords**: shrimp, aquaculture, nutritional programming, nutrition