Assessment of protein digestive capacity and utilisation during ontogeny of Senegalese sole larvae: a tracer study using in vivo produced radiolabelled peptide fractions

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Ghent University, Belgium, 2-5 September 2013
ASSESSMENT OF PROTEIN DIGESTIVE CAPACITY AND UTILISATION DURING ONTOGENY OF SENEGALESE SOLE LARVAE: A TRACER STUDY USING IN VIVO PRODUCED RADIOLABELLED PEPTIDE FRACTIONS

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Introduction

• Major challenge in larviculture:
  - Optimisation of inert diets (fit requirements)

• Digestive system not matured at first-feeding:
  - Seem to have difficulties in digesting complex proteins

• Examples of digestive proteases activities in Sole:

More tools needed for a reliable assessment of larval protein digestive capacity

Engrola et al. 2009
Objectives

1- Develop a method allowing the production of radiolabelled peptides and proteins of different MW

- *In vivo* method; *Artemia* system
- Size exclusion chromatography
Objectives

1- Develop a method allowing the production of radiolabelled peptides and proteins of different MW

- *In vivo* method; *Artemia* system
- Size exclusion chromatography

2- Use these tools to study the development of the protein digestive capacity and metabolic utilisation during the ontogeny of fish larvae

- Controlled tube-feeding methodology
- Senegalese sole larvae
Characterisation of the MW distribution of the Artemia protein content

- Methodology -

• Artemias production and protein extraction:

- Artemias EG decapsulated cysts
- 35%, 30°C, 60L, 200 art/ml
- Hatching (0h)
  - 15 hours
  - 24 hours
  - 36 hours
- Triplicates
  - Harvesting
  - Snap freezing
  - Freeze-drying
- Protein extraction
  - 100 mM PIPES, 1 mM EGTA, 1 mM MgCl2, pH 6.5, protease inh. mix (Oulton et al. 2003)
  - Grinding
- Size exclusion chromatography
Characterisation of the MW distribution of the Artemia protein content

- Methodology -

**Concentration**
- 3 kDa
- Centrifugal filter

**Artemia protein extract**

**High MW**

**Size exclusion chromatography**
- on HiLoad 26/60 Superdex 200 prep grade column (GE)
- Separation range: 600-10 kDa
- 100 mg protein per injection
- Elution with extraction buffer
- Absorbance monitoring at 280 nm
- Elution flow rate: 2 ml/min
- Fractions collection: 2 ml
Characterisation of the MW distribution of the Artemia protein content

- Methodology -

**Concentration**
- 3 kDa
- Centrifugal filter

**Size exclusion chromatography**
- on HiLoad 26/60 Superdex 200 prep grade column (GE)
- Separation range: 600-10 kDa
- 100 mg protein per injection
- Elution with extraction buffer
- Absorbance monitoring at 280 nm
- Elution flow rate: 2 ml/min
- Fractions collection: 2 ml

**Artemia**
- protein extract

**High MW**

**Concentration**
- Partial freeze-drying

**Size exclusion chromatography**
- on Superdex™ Peptide 10/300 GL column (GE)
- Separation range: 7-0.1 kDa
- 10 mg protein per injection
- Elution with extraction buffer
- Absorbance monitoring at 280 nm
- Elution flow rate: 0.5 ml/min
- Fractions collection: 0.5 ml

**Low MW**

Characterisation of the MW distribution of the Artemia protein content

- Results -

- High MW protein distribution analysis:

  - Hatching (0h)

  - 15 hours

  - 24 hours

  - 36 hours
Characterisation of the MW distribution of the Artemia protein content

- Results -

- High MW protein distribution analysis:

  - Hatching (0h)
  - 15 hours
  - 24 hours
  - 36 hours
Characterisation of the MW distribution of the Artemia protein content

- Results -

• High MW protein distribution analysis:

<table>
<thead>
<tr>
<th>Time</th>
<th>MW Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>&gt;500 kDa (Hatching)</td>
</tr>
<tr>
<td>15h</td>
<td>~ 500-130 kDa</td>
</tr>
<tr>
<td>24h</td>
<td>~ 130-30 kDa</td>
</tr>
<tr>
<td>36h</td>
<td>~ 30-10 kDa</td>
</tr>
</tbody>
</table>

Diagram showing absorbance at 280 nm (AU) against elution volume (ml) for different time points: 0h, 15h, 24h, and 36h.
Characterisation of the MW distribution of the Artemia protein content

- Results -

- High MW protein distribution analysis:

- Hatching (0h):
  - >500 kDa
  - ~500 - 130 kDa
  - ~130 - 30 kDa
  - ~30 - 10 kDa

- 15 hours:
  - >500 kDa
  - ~500 - 160 kDa

- 24 hours:
  - ~160 - 10 kDa
  - ~500 - 160 kDa

- 36 hours:
  - >500 kDa
  - ~500 - 160 kDa
  - ~160 - 10 kDa
Characterisation of the MW distribution of the Artemia protein content

- Results -

- Low MW peptides distribution analysis:

![Graphs showing MW distribution analysis at different time points: Hatching (0h), 15 hours, 24 hours, 36 hours.](image-url)
**Characterisation of the MW distribution of the Artemia protein content**

- **Results** -

- Low MW protein distribution analysis:

![Graph showing protein distribution at different time points with MW values: 15 hours (~1.9-0.5 kDa), 24 hours (~1.9-0.36 kDa), 36 hours (~1.9-0.36 kDa)](image_url)
Characterisation of the MW distribution of the Artemia protein content

- Results -

- Low MW protein distribution analysis:

![Graphs showing protein distribution at different time points: Hatching (0h), 15 hours, 24 hours, 36 hours.](image)
In vivo production of radiolabelled proteins and peptides of different MW

- Methodology -

[14C]-AA mixture (1 mCi)

Artemias EG decapsulated cysts

35%, 30°C, 60L, 200 art/ml

At hatching

2.5 L, 200 art/ml

Air supply

Trap 1, 1 L, 0.5 M KOH

Trap 2
In vivo production of radiolabelled proteins and peptides of different MW

**Methodology**

At hatching, Artemias EG decapsulated cysts are exposed to a [\(^{14}\)C]-AA mixture (1 mCi) for 16 hours of labelling. The labelled Artemias are then separated using air supply and trapping systems (Traps 1 and 2) with 1 L of 0.5 M KOH. Morais et al. 2004

![Graph showing the time course of radiolabelled proteins and peptides](image)
In vivo production of radiolabelled proteins and peptides of different MW

- Methodology -

At hatching

Artemias EG decapsulated cysts

[\(^{14}\text{C}\)]-AA mixture (1 mCi)

At hatching

2.5 L 200 art/ml

16 hours of labelling

Percentage of whole system radiolabel in each compartment:

- Artemia: 32.1%
- Water: 33.0%
- Trap 1: 34.4%
- Trap 2: 0.5%
In vivo production of radiolabelled proteins and peptides of different MW

**Methodology**

- At hatching Artemias EG decapsulated cysts
- [\(^{14}\)C]-AA mixture (1 mCi) for 16 hours of labelling
- Harvesting
  - Lyophilisation
  - Protein extraction
  - Sample preparation (as previously)
- Radiolabelled proteins and peptides fractions of different MW
- Size exclusion chromatography
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

Methodology

- Rust et al. 1993; Rønnestad et al. 2000, 2001

Live preys

- 2 DAH

Microdiet

- 18 DAH

- 34 DAH

Artemia meal of 30 min before tube-feeding

- 12 DAH

- 21 DAH

- 34 DAH

[14C]-AA mix

- [14C]-1.0 kDa

- [14C]-6.8 kDa

n=6

Rust et al. 1993; Rønnestad et al. 2000, 2001
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

- Methodology -

Drawing by S. Tonheim & I. Rønnestad

24 hours incubation in the chambers
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

- Methodology -

Incubation seawater

Air supply

H^+

0.5 M KOH

24 hours incubation in the chambers

14C

14CO_2 trap (Catabolism)

Larval body (Retention)

Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

Methodology

- Larval body (Retention)
- 14C CO_2 trap (Catabolism)
- Incubation seawater (Evacuation)
- 0.5 M KOH
- Air supply

Drawing by S. Tonheim & I. Rønnestad
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

- Results -

Proportion of label (% of total tube-fed label) absorbed and evacuated by the larvae

1.0 kDa peptides

- Absorption (% total) - Evacuation (% total)

12 DAH  21 DAH  34 DAH

1.0 kDa peptides: high absorption (77-88%)
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

- Results -

Proportion of label (% of total tube-fed label) absorbed and evacuated by the larvae

6.8 kDa peptides

- Absorption (% total)
- Evacuation (% total)

- 6.8 kDa peptides: absorption increases along ontogeny
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

- Results -

Proportion of label (% of total tube-fed label) absorbed and evacuated by the larvae

6.8 kDa peptides

Absorption (% total)        Evacuation (% total)

12 DAH: 34% 17%  
21 DAH: 44% 23%  
34 DAH: 55% 31%

6.8 kDa peptides: absorption increases along ontogeny
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

- **Results** -

Proportion of absorbed label:  
- Retained (body)  
- Catabolised (trap)

1 kDa peptides

- 1.0 kDa peptides: retention efficiency constant along ontogeny (63-71%)
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

- Results -

Proportion of absorbed label: - Retained (body)  
- Catabolised (trap)

6.8 kDa peptides

- 6.8 kDa peptides: retention efficiency tends to increase along ontogeny
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

- Results -

Proportion of absorbed label:
- Retained (body)
- Catabolised (trap)

6.8 kDa peptides

ANOVA: $p=0.067$

- 12 DAH: 50% retention, 50% catabolism
- 21 DAH: 62% retention, 38% catabolism
- 34 DAH: 70% retention, 30% catabolism

- 6.8 kDa peptides: retention efficiency tends to increase along ontogeny
Protein digestive capacity and metabolic utilisation during the ontogeny of sole larvae

- Results -

Proportion of absorbed label: - Retained (body)
- Catabolised (trap)

\[ \text{6.8 kDa peptides} \]

Spearman’s correlation: \( \rho = 0.57, p = 0.02 \)

ANOVA: \( p = 0.067 \)

\( \text{\(^{14}\text{CO}_2\) Trap} \)
Conclusions

- **Characterisation of Artemia protein content:**
  - From 0 to 24 h: high content in protein > 500 kDa, ~ 7-2.8 kDa, 1.9-0.5 kDa
  - At 36 h: shift from high MW proteins to smaller ones

- **Use of produced radiolabelled peptides fractions through Artemia radiolabelling:**
  - Peptides of 1 kDa: - high absorption rate at all stages
  - constant retention efficiency at all stages
  - Peptides of 6.8 kDa: - poorly absorbed by 12 DAH stage
  - digestibility increases along ontogeny
  - body retention tends to increase along ontogeny

This method may be useful in defining the most suitable protein-ingredient types and MW range, for different developmental stages of Senegalese sole and other fish larvae
Conclusions

Linking weaning success to larval digestive capacity using radiolabelled peptide fractions

Introduction
- Larval digestive tract maturation and protein metabolism can be elucidated by techniques of intestinal digestion. Fish larvae have difficulties in digesting complex proteins.
- Peptides are thought to be the most important substrates for larval nutrition. Therefore, labelling Artemia with radiolabeled amino acids could be used as a tool to evaluate weaning transitions.

Labelling Artemia
- Radiolabelled Artemia could provide information on the digestive efficiency of the larvae.

Feeding plan
- The feeding plan involves providing radiolabelled Artemia to the larvae in order to determine their digestive efficiency.

Sole tube-feeding
- The Sole tube-feeding technique involves feeding radiolabelled Artemia to the larvae through the intestine.

Acknowledgments
- The authors acknowledge the financial support from the Portuguese Foundation for Science and Technology (FCT) and the Centre for Marine Sciences (CIM).
Acknowledgements

Study co-funded by:

• Project HYDRAA - PTDC/MAR/71685/2006
• Project EPISODE - PTDC/MAR/110547/2009
• Project MICALA – I&DIT Co-Promocão N°13380 (Portugal, supported by POAlgarve 21, QREN and European Union)

FCT post-doctoral grants:

• SFRH/BPD/65578/2009 (NR)
• SFRH/BPD/49051/2008 (SE)

Thanks for your attention!