Optimum phospholipids and antioxidant levels in microdiets for gilthead seabream larvae

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• Material and methods
• Effect of level and type of PL
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• Combined effect of PL and Se
• Conclusions
**Introduction**

- **Source of fatty acids** (Izquierdo & Koven 2010) and energy (Rainuzzo et al., 1992)
- **Lipoproteins synthesis** (Liu et al., 2002, Hadas et al., 2003)
- **Reduce malformations** (Kanazawa et al., 1981, Guerden et al., 1998; Cahu et al., 2003)
- **Feeding activity** (Koven et al., 1998)
- **Emusifiers** (Olsen & Ringø, 1997)
- **Digestion and transport of lipids** (Izquierdo et al., 2001; Morais et al., 2005), gut maturation (MacQueen Leifson et al., 2003; Wold et al., 2007; Saleh et al., 2012)
**Introduction**

<table>
<thead>
<tr>
<th>Species</th>
<th>Phospholipid supplemented</th>
<th>Optimal requirement</th>
<th>Reference</th>
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<tr>
<td>Common carp</td>
<td>Hen egg lecithin</td>
<td>2%</td>
<td>Geurden et al. (1995)</td>
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<tr>
<td>Red seabream</td>
<td>soybean lecithin (SBL)</td>
<td>5%</td>
<td>Kanazawa et al. (1983a)</td>
</tr>
<tr>
<td>Knife jaw</td>
<td>SBL</td>
<td>7.4%</td>
<td>Kanazawa et al. (1983b)</td>
</tr>
<tr>
<td>Japanese flounder</td>
<td>SBL</td>
<td>7%</td>
<td>Kanazawa (1993)</td>
</tr>
<tr>
<td>European sea bass</td>
<td>SBL</td>
<td>12%</td>
<td>Cahu et al. (2003)</td>
</tr>
<tr>
<td>Seabream</td>
<td>SBL</td>
<td>15%</td>
<td>Seiliez et al. (2006)</td>
</tr>
</tbody>
</table>
**Introduction**

- **AOE**: Antioxidant Oxidation Enzymes
- **ROS**: Reactive Oxygen Species
- **SOD**: Superoxide Dismutase
- **Se**: Selenium
- **CAT**: Catalase
- **GR**: Glutathione Reductase
- **GPX**: Glutathione Peroxidase
- **GSSG**: Glutathione Disulfide
- **2GSH**: Reduced Glutathione
- **H₂O₂**: Hydrogen Peroxide
- **H₂O**: Water
- **Fe²⁺**: Ferrous Iron
- **Fe³⁺**: Ferric Iron
- **H₂O**: Water
- **O₂**: Oxygen
- **Asc**: Ascorbic Acid
- **Asc-H**: Ascorbate
- **α-TOH**: α-Tocopherol
- **·OH**: Hydroxyl Radical

Betancoret al., Larvi 2013
Marine fish larvae are under a high oxidation risk

- High metabolic rate & oxygen requirements
- High water content and water reabsorption at metamorphosis
- High PUFA requirements
- Lipid content and lipid mobilization from yolk sac
- Feed with high surface/volume
- Long water exposure of feed...
Introduction

Objectives

1. How much? Which type? How will it affect digestive enzymes? How will it affect skeleton development? How will it affect oxidative status?

2. Will PL requirements be affected by the antioxidant vit E?

3. Se levels improve the performance of larvae fed optimum PL levels?
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Materials & Methods

- Tank volume 200 l
- Water renewal 25%/h
- Aeration 125 ml/min
- Photoperiod 12L:12D
- 1000-2000 lux
- Feeding 2.5-5 g/daily
- 2100 Seabream larvae 14 dph
  (T.L. 5 mm & 100 µg DBW)
Zootechnical Parameters

Total length, Dry body weight, Survival, Handling stress Test, Feed acceptance

Exp 1. Optimum krill phospholipids
Exp 2. Optimum soy phospholipids
Exp 3. Comparison between KPL or SBL
Exp 4. Combined PL/vit E levels
Exp 5. Ranged Se levels

Sparus aurata

Materials & Methods
Materials & Methods

Ingredients, feeds and larvae

- **Protein** (AOAC, 1995)
- **Moisture & Ash** (AOAC, 1995)
- **TBARS** (Burk et al., 1980)
- **Selenium Collision/reaction cell ICP-MS**

**Total Lipids** (Folch et al., 1957)

- **Neutral & polar lipids** (Olsen & Henderson, 1989)
- **Fatty Acids** (Christie, 1982, Izquierdo et al., 1990)
Materials & Methods

Digestive Enzymes

- Alkaline Phosphatase (Gee et al., 1999)
- Trypsin (Rotllant et al., 2008)
- Lipase (Rotllant et al., 2008)
- Phospholipase A2 (Huang et al., 2006)
Materials & Methods

**Whole mount staining**

Deformities studied according to Boglione et al. (2001)

Alizarin red (Izquierdo *et al.*, 2012, modified from Vandewalle *et al.*, 1998)
Gene expression (RT-PCR)

AOE markers
- CAT
- SOD
- GPX

Osteo markers
- BMP4
- RUNX2
- Alkaline phosphatase
- Osteocalcin
- Osteopontin
- Osteonectin
- Matrix Gla

Means compared by Duncan’s test (P < 0.05) using SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).
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Exp. 1 Krill phospholipids

Final Survival

High survival & stress resistance with 9-10% KPL

Survival after handling stress

Lower requirements than previous authors for European seabass using SBL (Cahu et al., 2003)

Saleh et al., 2012. Aquaculture Nutr.
Exp 2. Soybean lecithin levels

Final Survival

Poor effect of SBL on survival and stress resistance

Survival after handling stress

Up to 12% SBL did not affect pikeperch larval survival (Hamza et al., 2008)

**Exp 3. Krill phospholipids vs soybean lecithin**

**Final Survival**

Better survival and stress resistance by KPL than SBL even at lower dietary levels

**Survival after handling stress**

Krill phospholipid as a good PL source (Betancor et al. 2012)

\[ y = 2.1073 \ln(x) + 4.4686 \]

\[ R^2 = 0.744 \]

**Exp 3. Krill phospholipids vs soybean lecithin**

Better total length and body weight by KPL than SBL even at lower dietary levels

Higher effectiveness of marine PL (Salhi et al. 1999; Izquierdo et al. 2001; Wold et al. 2007)

**Exp. 1 Krill phospholipids**

\[ y = 131.46 \ln(x) - 5.6182 \]

\[ R^2 = 0.97623 \]

**Exp. 2. Soybean phospholipids**

\[ y = -4.6463x^2 + 86.934x - 102.84 \]

\[ R^2 = 0.8067 \]

*Stronger positive effect of KPL on Alkaline phosphatase and thus on maturation of digestive tract could contribute to the better growth*

*Saleh et al., 2012 & 2013. Aquaculture Nutr.*
Very similar effect of both PL on Trypsin activity, so... not related with the HUFA content of the PL source

**Exp. I Krill phospholipids**

\[ y = 158,61\ln(x) - 101,58 \]

\[ R^2 = 0,91999 \]

**Exp 2. Soybean lecithin levels**

\[ y = 109,01\ln(x) + 104,27 \]

\[ R^2 = 0,56297 \]

Stronger effect of KPL on Neutral lipase activity

Neutral lipase has a high affinity for n-3 HUFA esterified lipids (Izquierdo et al., 2002)

Exp. I Krill phospholipids

$y = 513,78 \ln(x) - 113,95$

$R^2 = 0.869$

Exp. 2. Soybean lecithin levels

$y = 45,662 \ln(x) - 35,652$

$R^2 = 0.8521$

Very similar effect of both PL sources on PLA2... only depending on the level of PL


Transcriptional regulation of PLA2 by dietary PL (Zambonino-Infante and Cahu, 1999). SBL enhance gut and liver lipid transport activity (Liu et al., 2002)
**Exp 3. Krill phospholipids vs soybean lecithin**

Both PL enhanced bone mineralization, but KPL was more effective and at 7% reduced bone anomalies.

**Saleh et al, in press. Aquaculture Nutr.**
Intramembranous bone

Endochondral bone

**IGF**

**Sox9**

**Runx2**

**PPARY**

**BMP-2**

**Collagen I**

**Osteopontin**

**Osteocalcin**

**Matrix GLA**

**ALP**

**chondroblast**

**chondrocyte**

**adipocyte**

**osteoblasts**

**osteocytes**

**Mesenchymal stem cell**

**cartilage**
Exp 3. Krill phospholipids vs soybean lecithin

At 30 dph OP (SPP1) is more sensitive to DHA levels than in other bone molecular marker studied (Izquierdo unpublished data).

The three mineralization protein genes were up-regulated by KPL and to a lower extent by SBL, but Osteopontine expression was better correlated with the mineralization observed.

Exp 3. Krill phospholipids vs soybean lecithin

N-3HUFA and DHA up-regulates BMP4 and OC in seabream larvae (Izquierdo et al., 2012)

BMP was up-regulated by KPL and it was negatively related to lordosis incidence. The other two markers of early and late bone differentiation were also affected by KPL

**Exp 3. Krill phospholipids vs soybean lecithin**

TBARs

7 and 9% SBL and 9% KPL increased ROS production in the larvae

Exp 3. Krill phospholipids vs soybean lecithin

AOE, particularly CAT, gene expression was up-regulated by SBL and 9% KPL

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Exp 4. Combined Vit E and PL levels

Vit E addition was not able to improve survival or growth in SBL diets

Dietary vit E improved growth in KPL diets

Exp 4. Combined Vit E and PL levels

Increased dietary Vit E raised the percentage of mineralized bone in a given size class.

Vit E addition did not affect any of the 7 bone biomarkers studied, although KPL and, thus n-3 HUFA, up-regulated them, but not through ROS!!

Exp 4. Combined Vit E and PL levels

Exp 4. Combined Vit E and PL levels

CAT gene expression is better correlated than GPX to oxidative risk by DHA (Izquierdo et al., 2012).

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Addition of Se up to 8-12 mg/kg in diets containing 9% KPL increased larval survival and stress resistance.

Exp 5. **Effect of Se levels**

Larval Se and n-3 HUFA were correlated

R² = 0.82

R² = 0.86

Larval fatty acids %

N-3HUFA

DHA

(μg Se/g larvae)*1000

Dietary and larval Se was negatively correlated with TBARs and AOE gene expression.

Antioxidants may interact with cellular receptors and transcriptional factors that may further lead to changes in mRNA levels (Olsvik et al. 2011).

Dietary and larval Se, or reduction in ROS was better correlated with ALP gene expression than the other 6 bone molecular markers.

In agreement, there was an increased mineralization and reduced scoliosis incidence (Poster session, Bénitez et al.)

Oxidative balance in marine larvae

Pro-oxidants
- ROS
- PUFA
- Fe
- Stress
- Light
- Photoperiod
- Canibalism

Anti-oxidants
- Vit C
- α-TOH
- Se
- Carotenoids
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1. Seabream from 16-45 dph require about 9-10% HUFA rich PL to completely substitute live preys. KPL improves digestion, transport and deposition of dietary lipids and contribute to reduce skeleton anomalies by up-regulating bone molecular markers, particularly OP and BMP, inducing early mineralization and resistance of vertebral bodies to reduce anomalies such as lordosis and kyphosis.

2. SBL is not able to promote survival and growth as effectively as KPL, increases oxidative risk in the larvae and up-regulates AOE genes.

3. Dietary α-tocopherol promoted the beneficial effects of dietary PL, promoting growth, denoting its protective role against oxidation and reducing larval TBARs and gene expression of SOD and CAT. In relation to oxidative risk by HUFA, CAT gene expression is a better molecular marker.

4. Increase Se up to 8-12 mg/kg improved larval survival and stress resistance, protecting the larval tissues from oxidative risk
Acknowledgments