New insights of the effects of dietary vitamin A on flatfish skeletogenesis: the case of *Solea senegalensis*

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Fish Nutrition and Larval Rearing Group

IRTA – Sant Carles de la Ràpita; Spain

Larvi’09. Ghent, September 2009
Main flatfish world aquaculture production

<table>
<thead>
<tr>
<th>World aquaculture production</th>
<th>in Tones</th>
<th>in Thousands US $</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flatfishes</td>
<td>7%</td>
<td>12%</td>
</tr>
<tr>
<td>Total marine fish</td>
<td>1737,080</td>
<td>5884,840</td>
</tr>
</tbody>
</table>

Production increase (2000-06)

<table>
<thead>
<tr>
<th>Europe</th>
<th>Production 100%</th>
<th>Value in Thousands US $ 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>World</td>
<td>400%</td>
<td>100%</td>
</tr>
<tr>
<td>Flatfish aquaculture production problems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Low quality and discontinuous spawnings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Howell et al., 2009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Mortality by pathogen infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zarza et al., 2003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Pigmentary disorders</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamre et al., 2007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• High incidence of skeletal deformities and abnormal eye migration (40-90%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aritaki et al., 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Takeuchi et al., 1998</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gavaia et al., 2002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lewis and Lall, 2006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fernández et al., 2009</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Flatfish larvae development

1. Metamorphosis in flatfish: transformation, from symmetrical to asymmetrical anatomy, during development
   - 90º rotation in body position.
   - One eye migrate to the ocular upper side.
   - Formation of pseudomesial bone??
   - Settlement: the change from pelagic to benthic habitat.

2. Metamorphosis is driven by thyroid hormones

3. During metamorphosis: flatfish axial skeleton ossification, where the most severe skeletal deformities are located

From Fernandez-Diaz et al., 2001 and Schreiber 2006.
### Flatfish larvae development species-characteristics

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Hatch</th>
<th>First feeding (PH)</th>
<th>Metamorph start (PH)</th>
<th>Metamorph End (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japanese flounder</td>
<td>63h 40°C day</td>
<td>4 days 60°C day</td>
<td>12-14days 210°C day</td>
<td>40 days 525°C day</td>
</tr>
<tr>
<td>Summer flounder</td>
<td>48-60h 36-45°C day</td>
<td>23 days 425 °C day</td>
<td>35 days 647°C day</td>
<td>65 days 1170°C day</td>
</tr>
<tr>
<td>Atlantic halibut</td>
<td>15 days 70°C day</td>
<td>&lt;50 days 265°C day</td>
<td>81-90 days 1035°C day</td>
<td>135 days 1552°C day</td>
</tr>
<tr>
<td>Senegal sole</td>
<td>24-48h 34°C day</td>
<td>3 days 51°C day</td>
<td>10 days 170°C day</td>
<td>20 days 340°C day</td>
</tr>
</tbody>
</table>

**General patterns of metamorphosis:**

**Plaice-like:** metamorphosis at larger sizes (Atlantic halibut)

**Sole-like:** metamorphosis at smaller sizes and short duration (Senegal sole)

1. Egg size ranges: 0.5 and 4.25 mm

2. Bigger eggs ➡️ bigger larvae ➡️ longer developmental time

4. Different number of skeletal structures such as vertebral bodies (Atlantic halibut 50 versus Senegal sole 45).

Osse and Van der Boogaart, 1997
Different factors that affect bone development

<table>
<thead>
<tr>
<th>Xenobiotic Factors</th>
<th>Abiotic factors</th>
<th>Biotic factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Contaminants</td>
<td>• Light</td>
<td>• Culture density</td>
</tr>
<tr>
<td>• Pesticides</td>
<td>• pH</td>
<td>• Pathogens</td>
</tr>
<tr>
<td>• Herbicides</td>
<td>• CO₂, O₂</td>
<td>• Genetics</td>
</tr>
<tr>
<td>• Heavy metals</td>
<td>• Temperature</td>
<td>• Nutrition</td>
</tr>
<tr>
<td>• Organochlorates</td>
<td>• Radiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Salinity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Water flow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Tank characteristics</td>
<td></td>
</tr>
</tbody>
</table>
Vitamin A roles during development

- Retinoids
- Proliferation
- Nervous system
- Skin
- Immune system
- Spermatogenesis
- Differentiation
- Embryogenesis
- Epithelia
- Bone and cartilage

Ross et al., 2000
Objective

Dietary VA effects on skeletogenesis:

The present work aims to review the literature related to the effects of dietary VA imbalance in flatfish skeletogenesis, and compare the results with recent ones in Senegalese sole.

1. Broodstock spawning performance and quality
2. Juveniles skeletogenesis
3. Larval performance and skeletogenesis
Japanese flounder broodstock-spawned egg quality

<table>
<thead>
<tr>
<th>Dietary VA (IU kg⁻¹)</th>
<th>NSD</th>
<th>CD</th>
<th>SD</th>
<th>ED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,566</td>
<td>11,000</td>
<td>56,333</td>
<td>337,000</td>
</tr>
</tbody>
</table>

- **NSD**: Feed intake decreased and spawning stopped for periods and higher number of eggs spawned.
- **CD**: Longer spawning period and higher fecundity, % buoyant eggs, and hatching rate.
- **ED**: Higher retinoid content, being retinol the most abundant contrary to wild and CD eggs where it was retinal.
- **SD**: Lower % of abnormal larvae and eggs, but no differences in % of abnormal larvae and survival rate.
- **No differences in % of abnormal larvae and survival rate.**

Furuta et al., 2001; 2003
Atlantic halibut-juveniles

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control</th>
<th>Low Phosphorus</th>
<th>Low Vitamin C</th>
<th>High Vitamin A</th>
<th>Oxidized Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormality</td>
<td>No. FA</td>
<td>No. of Fish</td>
<td>No. FA</td>
<td>No. of Fish</td>
<td>No. FA</td>
</tr>
<tr>
<td>A.</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0.67</td>
<td>1</td>
</tr>
<tr>
<td>B.</td>
<td>0</td>
<td>0</td>
<td>63</td>
<td>13.97</td>
<td>5</td>
</tr>
<tr>
<td>C.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>D.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>E.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F.</td>
<td>0</td>
<td>0</td>
<td>197</td>
<td>43.68</td>
<td>5</td>
</tr>
<tr>
<td>G.</td>
<td>3</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>0.44</td>
</tr>
<tr>
<td>H.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>I.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>J.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L.</td>
<td>0</td>
<td>0</td>
<td>186</td>
<td>41.24</td>
<td>5</td>
</tr>
<tr>
<td>M.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Q.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>451</td>
<td>42</td>
<td>85</td>
<td>139</td>
</tr>
</tbody>
</table>

### Abnormalities

- B: scoliosis
- C: vertebral fusion
- D: vertebral compression

#### Vertebrae

- Neural element abnormality: 0
- Hemal element abnormality: 0

### Dietary VA

- Retinyl acetate: 14 weeks
- Increased level in liver retinol content
- Liver fatty acid composition mean reduction of 14% in EPA and 8.2% in total n-3 fatty acid series in high VA diet fishes
- No craniofacial deformities

### Skeletal Deformities

- Main skeletal deformities: scoliosis, fused and compressed vertebra
- Up to 60% fishes presented scoliosis.

Lewis and Lall, 2009 (sub to J. Appl. Ichthy.)
Japanese flounder-larvae

Two different approaches

- **Hoxd-4 expression** was affected by > 0.1 µM RA treatment in a dose response manner
- **Implied pharyngeal arches development**
  (Suzuki et al., 1998).

- **atRA, 9-cis-RA and 13-cis-RA exposed larvae (6-9dph)** presented malformed lower jaw, vertebrae and caudal fin complex
- **Skeletal deformities were more severe with atRA exposure**
- **RA skeletal deformities underlies on RAR pathway**
  (Haga et al., 2003).

Balneation

- **RA treatment** depressed *hoxd-4* and *shh* expression
  - **malformed pharyngeal and trabecular cartilages**
  (Suzuki et al., 1999).

- **RA and disulfiram treatment affected to pharyngeal and pectoral skeleton**
  (Suzuki et al., 2000).

- **6-9dph larvae exposed to RAR- and RXR selective agonist (25nM) presented jaw deformities**
  (Haga et al., 2003).
Japanese flounder-larvae

Two different approaches

Balneation

Feeding

Live prey enrichment with VA

Larvae fed enriched VA live prey

VA palmitate

• Artemia metabolized atRA to another retinoid, but no isomerized RA
• When enriched with 200*10^6 IU kg^(-1) of different VA compounds, all others presented compressed vertebrae (Takeuchi et al., 1998). (Haga et al., 2006).
• A-B (3-19 dph) larvae fed enriched atRA rotifer presented highest incidence of skeletal (jaw and caudal vertebrae) and pigmentary abnormalities (Haga et al., 2002).
• Rotifiers accumulated more efficiently VA compounds (Giménez et al., 2007).
• Rotifers accumulated more efficiently VA compounds
• Retinyl acetate

Safe level (flounder skeletogenesis) of VA in Artemia less than 50,000 IU kg^(-1) enrichment (Haga et al., 2006).

Larvae isomerized RA

(200*10^6 IU kg^(-1) of different VA compounds, all others presented compressed vertebrae (Takeuchi et al., 1998). (Haga et al., 2006).

18-21 dph): max. accumulation of atRA at 3h post feeding, at 18h decreased in 80% (Sedi et al., 1995).

Rotifer: higher VA content when enriched in darkness

Artemia VA content peaks at 6-18h post isomerization (Haga et al., 2004) and 200*10^6 IU kg^(-1) of different VA compounds, all others presented compressed vertebrae (Takeuchi et al., 1998). (Haga et al., 2006).

- G larvae (27-31dph) fed Artemia enriched increasing VA palmitate levels induced hypermelanosis and vertebral deformities (Tarui et al., 2006)
### Summer flounder- pre-metamorphic larvae

<table>
<thead>
<tr>
<th>Balneation</th>
<th>atRA (nM)</th>
<th>atRA (nM)</th>
<th>atRA (nM)</th>
<th>atRA (nM)</th>
<th>atRA (nM)</th>
<th>9-cis-RA (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

10 days exposure

• No differences in growth and survival rate

• Increasing RA concentration increased % of fishes with hyperpigmentation

• The incidence of jaw, vertebral and fin deformities increased with RA concentration

• Fish length and skeletal deformities in jaw, pectoral fin were correlated

• atRA induced higher frequency of pectoral and vertebral deformities than 9-cis-RA

• Control fish: high level of skeletal deformities

Martínez et al., 2007
Atlantic halibut- larvae

<table>
<thead>
<tr>
<th></th>
<th>Enriched artemia</th>
<th>Zooplankton</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary VA content</td>
<td>466 IU kg⁻¹</td>
<td>66 IU kg⁻¹</td>
</tr>
</tbody>
</table>

45 days feeding

• No differences in growth and survival rate
• VA content 50% higher in zooplankton-fed fish
• Zooplankton-fed fish accelerated eye migration and higher % eye migrated larvae
• Zooplankton-fed fish had more mineralised bone.

Enriched Artemia and zooplankton were sources of different amounts of many other nutrients!!!

Moren et al., 2001; Hamre et al., 2002; Saele et al., 2003.
Senegalese sole-larvae

- Lower n° of thyroid follicles. 
- Higher size and T3 and T4 immunoreactivity with higher dietary content at 48 dph.
- Eye migration at 10 dph.
- 31 days feeding (Retinyl palmitate).
- Control
- T1
- T2
- T3
- 37,000
- 44,666
- 82,666
- 203,000

Fernández et al., 2009.
### Senegalese sole- larvae

<table>
<thead>
<tr>
<th>Artemia VA content (IU kg⁻¹)</th>
<th>Control</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37,000</td>
<td>44,666</td>
<td>82,666</td>
<td>203,000</td>
</tr>
</tbody>
</table>

#### 31 days feeding

**lavae 6 to 37 dph**

- Increasing VA dietary content increased % deformed fish
- Control fish presented high frequency of deformities
- No jaw deformities, and only cranial malformations in T3 (ceratohyal, ceratobranchial and operculum)
- Higher % of deformity in caudal vertebrae and caudal fin complex
- Bone ossification: different sensitivity to VA dietary imbalance

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Fernández et al., 2009.
Senegalese sole-larvae

Chondrocyte Apoptosis

MSC

Chond. Hip.

Osteoblast

Bone

Intramembranous

Fernández et al., 2009 (sub to J. Appl. Ichthy.)
Senegalese sole-larvae

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemia VA content (IU kg⁻¹)</td>
<td>37,000</td>
<td>44,666</td>
<td>4,496</td>
<td>12,911</td>
</tr>
</tbody>
</table>

31 days feeding

- Increasing VA dietary content

31 days feeding larvae 6 to 37 dph

- Control fish presented high frequency of deformities

- No jaw deformities, and only cranial malformations in T3 (ceratohyal, ceratobranchial and operculum)

- Higher % of deformity in Caudal vertebrae and caudal fin complex

- Bone ossification: different sensitivity to VA dietary imbalance

Fernández et al., 2009 (sub to J. Appl. Ichthy.)
Senegalese sole-larvae

VA ES content 1,500 IU kg\(^{-1}\) (Retinyl palmitate addition)

<table>
<thead>
<tr>
<th>Dietary treatments</th>
<th>Development larvae stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-metamorph</td>
</tr>
<tr>
<td></td>
<td>(3-10 dph) Rotifer</td>
</tr>
<tr>
<td>T1</td>
<td>Easy selco (ES)</td>
</tr>
<tr>
<td>T2</td>
<td>Easy Super Selco</td>
</tr>
<tr>
<td>T3</td>
<td>ES+ VA(x10)</td>
</tr>
<tr>
<td>T4</td>
<td>ES+ VA(x50)</td>
</tr>
<tr>
<td>T5</td>
<td>ES</td>
</tr>
<tr>
<td>T6</td>
<td>ES</td>
</tr>
<tr>
<td>T7</td>
<td>ES</td>
</tr>
<tr>
<td>T8</td>
<td>ES</td>
</tr>
</tbody>
</table>

Fernández et al., 2009 (in prep.)
Senegalese sole- larvae

VA ES content 1,500 IU kg$^{-1}$ (Retinyl palmitate addition)

- Increasing VA dietary content at early stages decreased survival rate
- Lower survival rate might increase food availability
- Only at early stages, increasing levels of VA increased mean nº of vertebral bodies
- Vertebral deformities increased with developmental time even after metamorphosis
- ESS might have some nutritional imbalance (caudal fin vertebrae)

Fernández et al., 2009 (in prep.)
Conclusions: Senegal sole

- Senegal sole larva seems to be very sensitive to dietary VA, as shown by the high frequency of skeletal deformities with increasing VA levels.

- Dietary VA affected thyroid follicles development.

- Chondral bones are more sensitive to VA dietary imbalance than intramembranous ones, however skeletal deformities in chondral bones were less severe in terms of fish quality.

- Less than 44,000 IU kg$^{-1}$ VA dietary content should be tested.
Conclusions: Flatfishes

1. We know how to induce skeletal deformities in flatfishes, but we still don’t know how to reduce them

2. Effects of high egg retinol content on larval quality remain unknown

3. Low VA dietary content stopped reproduction

4. Further experiments should be done to know optimum dietary VA level for broodstock

5. It is important to take into account the overall concentration of vitamin A in commercial feed

6. VA dietary imbalance could disrupt fish metabolism (fatty acids)

7. For optimal growth, VA content in halibut and flounder diets should be higher than 2,500 IU kg\(^{-1}\) (NRC), and around 8-9,000 IU kg\(^{-1}\) (Moren et al., 2001 and Hernandez et al., 2005), whereas for skeletogenesis less than 52,000 IU kg\(^{-1}\)

8. Attention should be paid to temperature, light and oxygen during live prey VA enrichment

9. VA dietary doses could accelerate ossification and metamorphosis processes, leading to reduce variability in size, and allowing to an early weaning
Conclusions:

9. **VA could reduce pigmented disorders (albinism), but at levels that could increase skeletal deformities and hyperpigmentation**

10. **VA dietary effects on skeletogenesis depends on:**
   - Ontogenic development of flatfish species
   - VA form used (RA highest power)
   - VA concentration
   - Nutritional approach used
   - Fish developmental stage

11. Less than 50,000 IU kg\(^{-1}\) might be the safe level for flounder skeletogenesis and less than 44,000 IU kg\(^{-1}\) for sole

12. **Further molecular and proteomic experiments should be done to identify RA regulation pathways related to bone formation and the appearance of skeletal deformities**

13. It is recommended to **standardize units of development** (\(^{\circ}\)C day, days start feeding, days post hatch or days post fertilization), **vitamin A form studied in diets, analysis methodology of VA content**, in order to facilitate the comparison of experimental results.
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