Environmental effects on the skeleton development in reared gilthead sea bream (Sparus aurata)

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ENVIRONMENTAL EFFECTS ON THE SKELETON IN REARED GILTHEAD SEABREAM (Sparus aurata)

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The skeleton differentiation, modeling and remodeling processes are under control of many factors:

- Genetic factors
  - Inner environment (i.e. mechano-regulatory pathway: Prendergast et al., 2007 — i.e. swim bladder, muscles, …; hormones, growth factors, …)
  - Outer environment (i.e. T, oxygen, nutrition, parasites, density, …)
- Environmental (non genetic) factors (i.e. epigenetics, environmental conditions)
Skeletal anomalies in fish juveniles represent one of the bottlenecks of current hatchery production

SA may arise throughout the entire life cycle

Economical issues:
- deformed fish induces distrust of aquaculture products in consumers
- fish must be periodically culled out for deformed individuals (60% of intensive production – FEAP data);
- deformed fish grow slower and sick (lower performances, higher sensitiveness to pathogens)

Ethical issue: welfare of cultured animals (CCRF, FAO 1995)
The present situation

- since the ‘70s, the same anomalies have occurred in juveniles
- no farm can boast of producing fish without anomalies
- incidences of SA are highly variable: the observed frequencies of fishes with more or less severe anomalies fluctuates between 15 and 100%
Major problems: the state-of-art on causative factors

✓ different effects of the same non-genetic factor on bone types and ossification, life stages, reared species, body regions, lots

✓ synergistic effect of different factors

✓ large availability of knowledge on human bone pathologies

BUT

the understanding of skeletal anomalies onset in reared fish is hampered by the fact that Teleosts present an exceptional diversity of skeletal tissues with respect to tetrapods.

Aims

quantitatively and qualitatively analyzing whether differences in skeletal elements (shape and number) arise and, in case they do, which are the most common:

1. when fish of different origins share the same environmental conditions (outer environment: Case 1 and 2)
2. when fish from the same eggs batch are reared under different larval rearing conditions (outer environment: Case 3)
3. when fish have or have not other non-skeletal anomalies (inner environment: Case 4)

in order

A. to individuate environmentally-induced skeletal anomalies;
B. to investigate if a relationship between those anomalies and skeletal bone tissues or types of ossification exists;
C. to identify the best practices for seabream larval rearing for obtaining lower deformity rates.
✓ **Studied species:** Gilthead seabream (*Sparus aurata*)

✓ **Total observed juveniles:** 2,079 juveniles (19 lots)
  - 288 wild-caught juveniles (3 lots)
  - 52 made up a lot of reared and wild juveniles (1 lot)
  - 1,739 reared juveniles (15 lots)

*In toto* double-staining for cartilage and bone (Dingerkus & Uhler, 1977; Park & Kim, 1984 modified) or X-rayed (Picker X-Ray cat 6191 – 805-E Control 599 Head)

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**Materials and methods**

Meristic counts
(vertebrae, rays and support elements of fin, pre-dorsal bones)

Skeletal anomalies
(Harder, 1975; Matsuoka, 1987; Schultze and Arrantia, 1989)
Analysis of skeletal anomalies in juveniles/subadults from different origin (wild vs reared) before and after restocking (7 months) in similar (Case I) or the same (Case II) semi-natural environmental conditions (*outer environment*).
Skeletal anomalies

<table>
<thead>
<tr>
<th>N individuals observed</th>
<th>WIIT07</th>
<th>WIIT06*</th>
<th>LVIT10</th>
<th>LVIT11*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total frequency (%) of malformed individuals</td>
<td>60.0</td>
<td>75.2</td>
<td>97.3</td>
<td>94.4</td>
</tr>
<tr>
<td>Average anomalies load</td>
<td>1.8</td>
<td>2</td>
<td>4.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Relative frequency (%) of individuals with at least one severe anomaly</td>
<td>0.0</td>
<td>2.2</td>
<td>33.3</td>
<td>22.4</td>
</tr>
<tr>
<td>Severe anomalies load</td>
<td>0</td>
<td>3.7</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Frequency (%) of severe anomalies observed on the total</td>
<td>0.0</td>
<td>6.8</td>
<td>14.0</td>
<td>15.7</td>
</tr>
</tbody>
</table>

* Recaptured lot

Results: Case 1
1. Opposite trend in the frequency of individuals affected by anomalies between recaptured reared and wild lots (*adaptive convergence*)

   reared $\rightarrow$ pressure on most severely deformed reared juveniles (selective mortality/ichthyophagous birds) $\rightarrow$ *adaptive convergence*

   wild $\rightarrow$ xenobiotic substances (?) + parasites (?) $\rightarrow$ *higher frequencies of some few anomalies*

2. The frequencies of individuals affected by anomalies of hypuralia (caudal fin) significantly increases in all recaptured lots, independently from the origin

*species-specific sensitiveness* (Boglione et al., 2001; Fernandez et al., 2008)
3. The frequencies of individuals affected by anomalies of arches, dorsal pterygiophores, dorsal and anal rays significantly decreases in all recaptured lots, independently from the origin

Hypotheses:

1) Negatively selected (valid if anomalous arches are associated with severe anomalies)? \(\rightarrow\) no negatively selected

2) Ossification process is influenced by environmental conditions (valid only if these skeletal elements share the same ossification processes)?
   \(\rightarrow\) no common processes neither embriiological origin

3) Normal hyperostosis processes (age-dependent) repaired/masked those anomalies?
   in intensive conditions, commercial size seabream show 1.9-78.9% of individuals with these anomalies \(\rightarrow\) extensive conditions favour bone repair (need for further deeper studies)
Case 3. Analysis of skeletal anomalies in juveniles from the same egg batch but reared under different larval environmental conditions (*outer environment*)

**Materials and Methods:**


### Intensive conditions

**3.1, 2 and 3 → Large Volume** (Cataudella et al., 2002), sampled at different age (3.3)

**3.4 → Mesocosms** (Divanach and Kentouri, 2000)

<table>
<thead>
<tr>
<th>Cases</th>
<th>Label</th>
<th>N</th>
<th>Characteristics</th>
<th>Origin</th>
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<tbody>
<tr>
<td>3.1</td>
<td>INIT06</td>
<td>55</td>
<td>Commercial juveniles reared in Valle Figheri hatchery (VE)</td>
<td>Intensive</td>
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<tr>
<td></td>
<td>LVIT01</td>
<td>66</td>
<td>Sister lot if INIT06 but reared in semi-intensive conditions</td>
<td>Large Volume</td>
</tr>
<tr>
<td>3.2</td>
<td>INIT07</td>
<td>123</td>
<td>Commercial juveniles reared in Valle Figheri hatchery (VE)</td>
<td>Intensive</td>
</tr>
<tr>
<td></td>
<td>LVIT02</td>
<td>122</td>
<td>Sister lot if INIT07 but reared in semi-intensive conditions</td>
<td>Large Volume</td>
</tr>
<tr>
<td>3.3</td>
<td>INIT19</td>
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</tr>
<tr>
<td></td>
<td>INIT18</td>
<td>105</td>
<td>Same lot of INIT19 at different age 85 dph</td>
<td>Large Volume</td>
</tr>
<tr>
<td></td>
<td>LVIT04</td>
<td>40</td>
<td>Sister lot if INIT19 but reared in semi-intensive conditions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LVIT05</td>
<td>105</td>
<td>Same lot of LVIT04 at different age 85 dph</td>
<td></td>
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<tr>
<td>3.4</td>
<td>INGR01</td>
<td>134</td>
<td>Commercial juveniles reared in HCMR (Iraklion, Crete, GR)</td>
<td>Intensive</td>
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<tr>
<td></td>
<td>MEGR04</td>
<td>128</td>
<td>Sister lot if INGR01 but reared in semi-intensive conditions</td>
<td>Mesocosm</td>
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</tbody>
</table>
**Meristic counts**

The intensively reared lots all showed a higher variability in meristic count than semi-intensive ones.

<table>
<thead>
<tr>
<th>Lots</th>
<th>Caudal fin</th>
<th>Anal fin</th>
<th>Dorsal fin</th>
<th>Pectoral fin</th>
<th>Pelvic fin</th>
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<tbody>
<tr>
<td></td>
<td>Vertebrae</td>
<td>Hyp.</td>
<td>Epur.</td>
<td>Lower rays</td>
<td>Pieryg.</td>
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<td>3-4</td>
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<td>7-8</td>
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<td>8-9</td>
<td>8-9</td>
<td>8-10</td>
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<td>8-9</td>
<td>8-10</td>
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<tr>
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<td>8-9</td>
<td>8-9</td>
<td>8-10</td>
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<td>8-9</td>
<td>8-10</td>
</tr>
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<td>5-6</td>
<td>2-4</td>
<td>8-10</td>
<td>8-9</td>
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<td>5-6</td>
<td>2-4</td>
<td>8-10</td>
<td>8-9</td>
</tr>
<tr>
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<td>2-4</td>
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<td>8-9</td>
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<td>2-4</td>
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<td>2-6</td>
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<td>5-9</td>
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<td>24-25</td>
<td>4-7</td>
<td>2-6</td>
<td>6-9</td>
<td>5-9</td>
</tr>
</tbody>
</table>

Results: Case 3

High variability in the number of vertebrae only in the intensive lots

- 4% → 23 Vert.
- 72% → 24 Vert.
- 1% → 26 Vert.
### Skeletal anomalies

<table>
<thead>
<tr>
<th>Group</th>
<th>Code</th>
<th>N. individuals</th>
<th>Frequency (%) of malformed individuals</th>
<th>Average anomalies load</th>
<th>Frequency of individuals with at least one severe anomaly (%)</th>
<th>Severe anomalies load</th>
<th>Frequency (%) of severe anomalies / total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>INIT06</td>
<td>55</td>
<td>100</td>
<td>8.6</td>
<td>74.5</td>
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<td>LVIT01</td>
<td>66</td>
<td>100</td>
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<td>54.5</td>
<td>1.3</td>
<td>19.5</td>
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<td>95.9</td>
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<td>52.8</td>
<td>1.6</td>
<td>23.2</td>
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<tr>
<td></td>
<td>LVIT02</td>
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<td>98.4</td>
<td>2.7</td>
<td>48.4</td>
<td>1.2</td>
<td>21.2</td>
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<td>105</td>
<td>96.2</td>
<td>5.8</td>
<td>47.6</td>
<td>1.6</td>
<td>13.9</td>
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<td>92.5</td>
<td>3.9</td>
<td>17.9</td>
<td>6.5</td>
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<td>1.8</td>
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<td>96.2</td>
<td>2.8</td>
<td>8.6</td>
<td>1.3</td>
<td>4.3</td>
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<td>Group 4</td>
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<td>95.5</td>
<td>4.2</td>
<td>28.4</td>
<td>1.5</td>
<td>10.5</td>
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<tr>
<td></td>
<td>MEGR04</td>
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<td>1.3</td>
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<tr>
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<td>1</td>
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<td>1.7</td>
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<td>5.8</td>
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<tr>
<td></td>
<td>WIIT04</td>
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<td>100</td>
<td>6.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>88</td>
<td>43.2</td>
<td>1.6</td>
<td>4.5</td>
<td>1.5</td>
<td>9.7</td>
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</tbody>
</table>
Skeletal anomalies

In order to understand why and how skeletal anomalies arise, the trend of meristic count variation and deformed individual rates was analyzed, taking into consideration the ossification typologies of the skeletal elements affected.

1. All the intramembranously ossified bones showed lower incidences of anomalies (and affected individuals) in semi-intensive rearing lots than in intensive one.

2. Skeletal elements ossifying on a pre-existing cartilaginous template did not always exhibit the same clear pattern, for instance showing a lower incidence of anomalies and lower count variability in all the Large Volumes lots but not in Mesocosm juveniles.
Qualitative analysis: CA

Binary matrix of data → frequencies matrix → CA (overall variance: 52.9%)

Results: Case 3

The same indicator’ colour specifies sister lots; semi-intensive lots are in green font
Quantitative analysis: NPMANOVA

**Group 1:** INIT06-LVIT01***

**Group 2:** INIT07-LVIT02***

**Group 3:** INIT19-LVIT04 *; INIT18-LVIT05***;
INIT19-INIT18***;
LVIT04-LVIT05 n.s.;

**Group 4:** INGR01-MEGR04 ***

*Differences are not significant only in LV lots sampled at different ages*

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*** = p≤0.001;
** = p≤0.01;
* = p≤0.05;
n.s. = not significant
I. Intensively reared lots showed higher variability in the vertebral number and higher frequencies of severely affected individuals: the 3.7% of the individuals show only anomalous vertebrae number vs the 12.7% of individuals with both anomalous vertebrae number AND fused vertebrae

→ fusion of vertebral bodies involve bone resorption and bone remodelling, as a primary pathology or in response to altered mechanical load (Kranenbarg et al., 2005; Witten et al., 2006)

→ vertebral fusions and changes in the number of vertebrae were attributed to a defect of notochord segmentation and disruption of vertebral centrum differentiation (Haga et al., 2009)

→ fused vertebrae show transdifferentiation of notochordal (at the intervertebral spaces) and periosteal (at the growth zone) cells into chondroblastic cells in compressed and fused vertebrae of Atlantic salmon, sea bass and gilthead seabream as a pathological response to a compressive mechanical environment (Beresford, 1981; Hall, 2005; Kranenbarg et al., 2005; Witten et al., 2005, 2009; Roberto, 2006; Fiaz et al., 2010).

A. This higher variability should be due to altered skeleton ossification processes (after embryonic development) → intensive rearing conditions (higher density, smaller water volumes) induce higher developmental instability

B. Higher selective pressure acting in semi-intensive conditions
2. The bones that undergone direct ossification (cranial bones, vertebrae and fin rays) showed lower incidences of anomalies and meristic variability in all the semi-intensive lots
   → intramembranous bones are less sensitive than chondral ones to nutrient deficiency or excess (Darias et al., 2010; Fernandez and Gisbert, 2010; Izquierdo et al., 2012)
   → Intramembranous ossification is highly dependent on environmental, non nutritional, factors?

3. Higher capacity of LVs than Mesocosm of augmenting the qualitative gap with the intensive sister lot: 5 out of the 6 anomalies that diminish only in LV affected bone that ossified indirectly
   → DHA levels in diet determine an augmentation of deformities in all skeletal elements with a cartilaginous precursor (Izquierdo et al., 2012)
   → Vit. C levels in food notably affect skeletal elements undergoing chondral ossification (Darias et al., 2010)

In Large Volumes the free entrance of wild plankton is allowed
   → higher nutritional value of live preys plays a positive effect on skeleton differentiation
4. **Inter-age differences between the two intensive lots were significant, but not between the two Large Volumes lots:**

- intensive rearing conditions induce higher developmental instability → many different developmental trajectories are then possible
  → *lower homeorhesis in intensive conditions with a consequent larger number of ‘allowed paths’*

- semi-intensive rearing methodology is an environment where a low number of ‘perturbations’ are present so enabling the maintenance of the main (species/stage-specific) developmental trajectory
  → *semi-intensive rearing methodology seems to be a system at higher homeorhesis with ‘canalised’ developmental trajectories*
**Case 4.** Effects on skeleton of the presence of anomalous and normal swim bladder in reared juveniles (*inner environment*)

287 juveniles (90 dph) were taken from Civita Ittica s.r.l. (Civitavecchia, RM, Italy) from the same rearing tank.

<table>
<thead>
<tr>
<th>Study</th>
<th>Label</th>
<th>N</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 4</td>
<td>SB</td>
<td>50 (17%)</td>
<td>presence of swim bladder</td>
</tr>
<tr>
<td></td>
<td>noSB</td>
<td>237 (83%)</td>
<td>absence of swim bladder</td>
</tr>
</tbody>
</table>
### Skeletal anomalies

* Results obtained not considering the anomaly 12

<table>
<thead>
<tr>
<th>Code</th>
<th>N. individuals</th>
<th>Frequency (%) of malformed individuals</th>
<th>Average anomalies load</th>
<th>Frequency of individuals with at least one severe anomaly (%)</th>
<th>Severe anomalies load</th>
<th>Frequency (%) of severe anomalies</th>
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<td><strong>This study</strong></td>
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<td>42.0</td>
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<td>100.0</td>
<td>5.8</td>
<td>100.0</td>
<td>1.8</td>
<td>17.2</td>
</tr>
<tr>
<td>NoSB*</td>
<td>237</td>
<td>100.0</td>
<td>9.7</td>
<td>48.5</td>
<td>1.7</td>
<td>8.7</td>
</tr>
</tbody>
</table>

- Anomalies charge resulted significantly different between the two lots ($p(same): 0.01722$, Mann-Whitney test)
- as many as 15 anomalies were observed only in NoSB seabream
- the most frequent anomalies in NoSB lot affected the pectoral fin (light anomalies) and haemal region (severe anomalies)
The difference between the two lots were qualitative and quantitative different

Many severe anomalies observed in NoSB lot were not found in SB:
- some axis deviations in certain regions (scoliosis, lordosis in pre-haemal vertebrae, kyphosis in haemal vertebrae)
- anomalous pre-haemal and haemal vertebrae bodies
- presence of calculi in the urinary ducts

- juvenile fish with uninflated swim bladder show difficulties in maintaining the level in the water column → overuse pectoral fins flapping (so increasing the activity of pre-haemal muscles) → more intense mechanical load on ossifying pre-haemal vertebrae → SA arise in pectoral fins and pre-haemal vertebrae at first, then in the haemal vertebrae
**outer environmental condition**

presence of an effective environmental effect on the skeleton processes (repair included)

**stocking density and tank volume** are powerful drivers in canalising the development trajectories of skeleton elements which ossify directly (without cartilaginous templates) in gilthead seabream larvae (*mechanical overload hypothesis*)

indirectly ossifying bones are more responsive to nutraceutical characteristics of administered **live preys** (*nutraceutical hypothesis*)

**inner environmental condition (uninflated swim bladder)**

*mechanical overload* caused by hyper-activity of pectoral fin muscles acting on differentiating skeletal elements located nearby the swim bladder (pre-haemal vertebrae).

It is possible to ameliorate the morphological quality of reared gilthead seabream juveniles by lowering the stocking densities (maximum 16 larvae/L), enlarging the volume of the rearing tanks in the hatchery (minimum 40 m³) and feeding larvae with a wide variety of live (wild) preys.

Larvae reared in such conditions showed low deformity rates surely up to pre-ongrowing phase
Acknowledgements

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Thank you for your attention!