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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

Environmental (non genetic) factors
(i.e. epigenetics, environmental conditions)

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,...)

Skeletal anomalies in fish juveniles

represent one of the bottlenecks of current hatchery production

SA may arise throughout the entire life cycle

Economical issues:

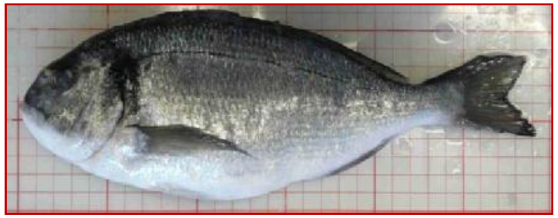
- ✓ deformed fish induces distrust of aquaculture products in consumers
- ✓ fish must be periodically culled out for deformed individuals (60% of intensive production – FEAP data);
- ✓ deformed fish grow slower and sick (lower performances, higher sensitiveness to pathogens)

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

The present situation

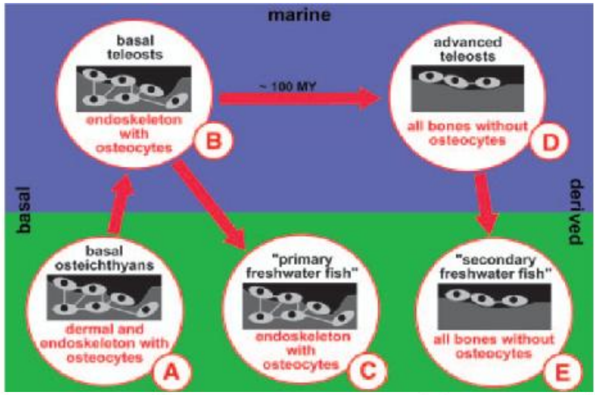
- ✓ since the '70s, the same anomalies have occurred in juveniles
- ✓ no farm can boast of producing fish without anomalies
- ✓ incidences of SA are highly variable: the observed frequencies of fishes with more or less severe anomalies fluctuates between 15 and 100%

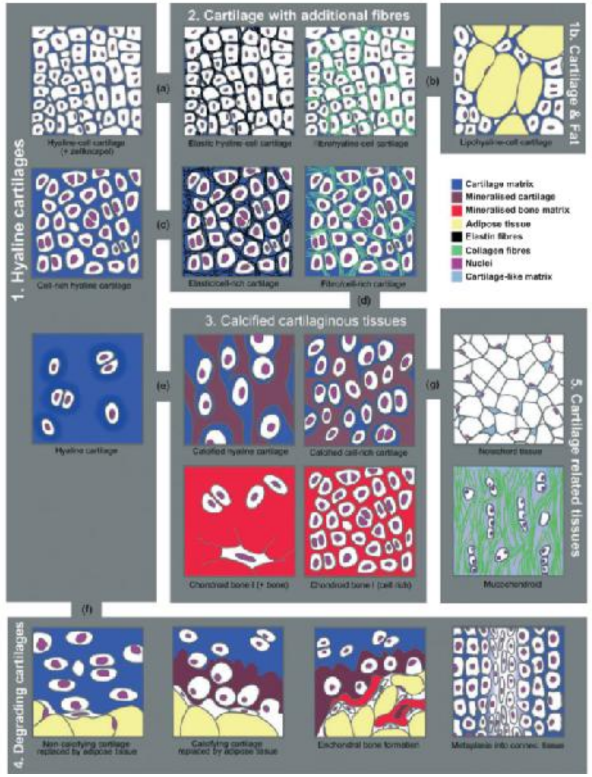




Major problems: *the state-of-art on causative factors*

Witten, P.E. & Huysseune, A. (2009). A comparative view on mechanisms and functions of skeletal remodelling in teleost fish, with special emphasis on osteoclasts and their function. Biol. Rev., 84, pp. 315–346





- ✓ 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.

Introduction

Aims

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

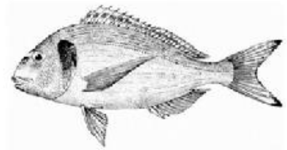
1. when fish of different origins share the same environmental conditions *(outer environment: Case 1 and 2)*
2. when fish from the same eggs batch are reared under different larval rearing conditions *(outer environment: Case 3)*
3. when fish have or have not other non-skeletal anomalies *(inner environment: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

Materials and methods

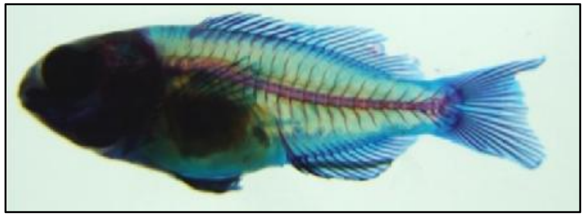
✓ **Studied species:** Gilthead seabream (*Sparus aurata)*



✓ **Total observed** juveniles**:** <u>2,079 juveniles (19 lots</u>)

- 288 wild-caught juveniles (3 lots)
- 52 made up a lot of reared and wild juveniles (1lot)
- 1,739 reared juveniles (15 lots)

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



Meristic counts

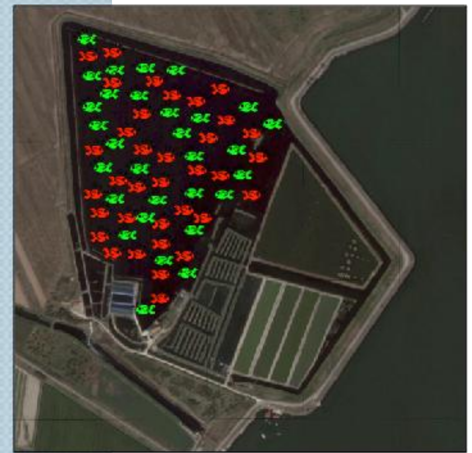
(vertebrae, rays and support elements of fin, pre-dorsal bones)

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)*

	Label	N	Characteristics	Origin
Case 1	LVIT10	264	Juveniles before stocking in Valle Morosina (VE)	Large Volume
	LVIT11	107	Same lot of LVIT10 but recaptured after 7 months in Valle	
	WIIT07	100	Juveniles before stocking in Valle Ghebo Storto (VE)	Wild
	WIIT06	137	Same lot of WIIT07 but recaptured after 7 months in Valle	



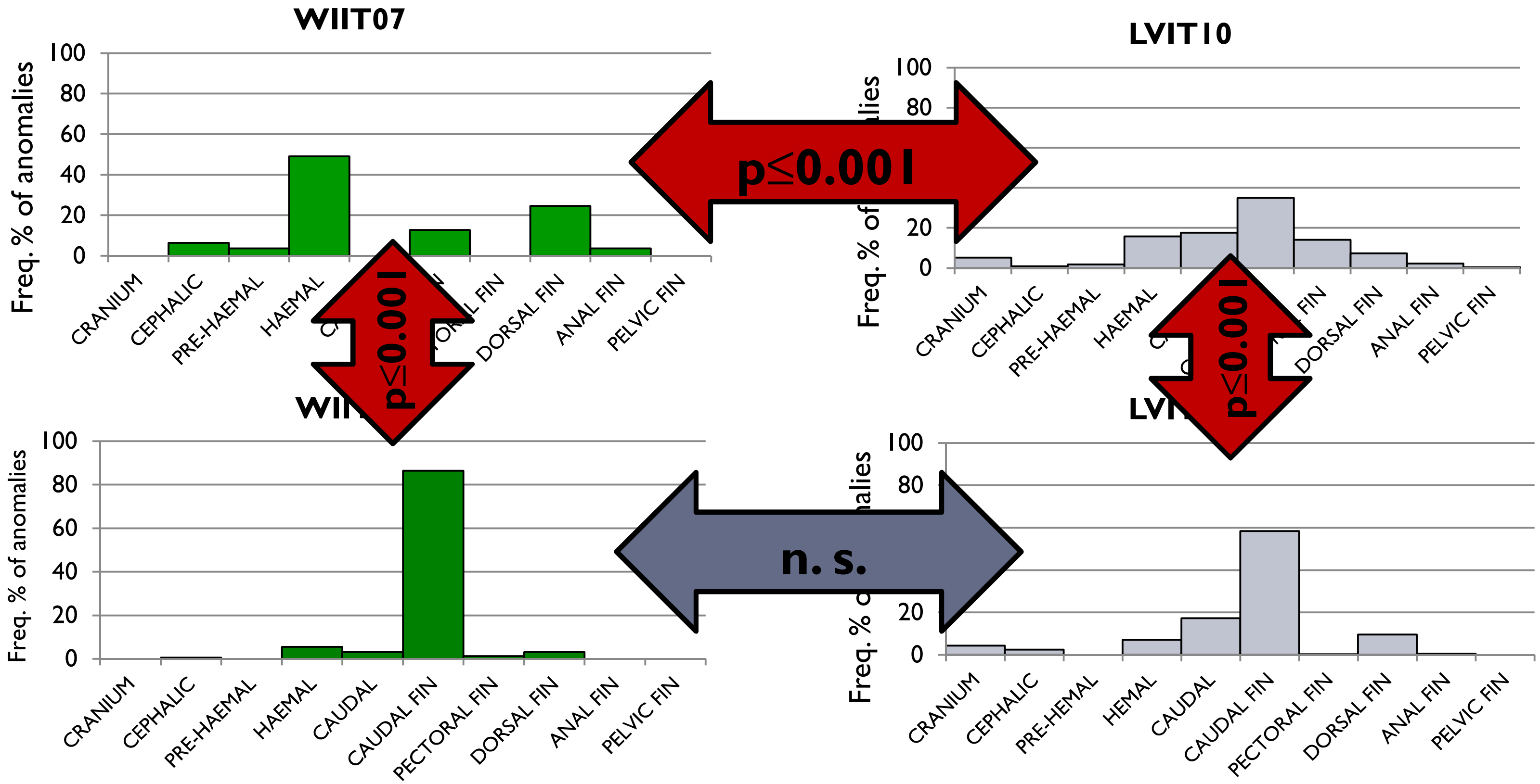
	Label	N	Characteristics	Origin
Case 2	INIT20	100	Reared juveniles before stocking in Valle Bonello (VE)	Intensive
	WIIT08	51	Wild juveniles before stocking in Valle Bonello (VE)	Wild
	MIXIT01	52	Recaptured lot after 7 months Valle Bonello (VE)	Mixed

Case I & II

Skeletal anomalies

* Recaptured lot

	WIIT07	WIIT06*	LVIT10	LVIT11*
N individuals observed	100	137	264	107
Total frequency (%) of malformed individuals	60.0	75.2	97.3	94.4
Average anomalies load	1.8	2	4.8	3.2
Relative frequency (%) of individuals with at least one severe anomaly	0.0	2.2	33.3	22.4
Severe anomalies load	0	3.7	1.9	2.1
Frequency (%) of severe anomalies observed on the total	0.0	6.8	14.0	15.7



Results and Discussion: Case I

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

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

wild → xenobiotic substances (?) + parasites (?) → *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) ? → no negatively selected

2) Ossification process is influenced by environmental conditions (valid only if these skeletal elements share the same ossification processes) ?

→ no common processes neither embriological 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 → 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)*

Intensive conditions

Semi-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)

Cases	Label	N	Characteristics	Origin
3.1	INIT06	55	Commercial juveniles reared in Valle Figheri hatchery (VE)	Intensive
	LVIT01	66	Sister lot if INIT06 but reared in semi-intensive conditions	Large Volume
3.2	INIT07	123	Commercial juveniles reared in Valle Figheri hatchery (VE)	Intensive
	LVIT02	122	Sister lot if INIT07 but reared in semi-intensive conditions	Large Volume
3.3	INIT19	105	Commercial juveniles reared in Valle Figheri hatchery (VE)	Intensive
	INIT18	105	Same lot of INI19 at different age 85 dph	
	LVIT04	40	Sister lot if INIT19 but reared in semi-intensive conditions	Large Volume
	LVIT05	105	Same lot of LVIT04 at different age 85 dph	
3.4	INGR01	134	Commercial juveniles reared in HCMR (Iraklion, Crete, GR)	Intensive
	MEGR04	126	Sister lot if INGR01 but reared in semi-intensive conditions	Mesocosm

Prestinicola, L., Boglione ,C., Makridis, P., Spanò, A., Rimator,i V., et al. (2013) Environmental Conditioning of Skeletal Anomalies Typology and Frequency in Gilthead Seabream (Sparus aurata L., 1758) Juveniles. PLoS ONE 8(2): e55736. doi:10.1371/journal.pone.0055736

Meristic counts

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

		Caudal fin				Anal fin		Dorsal fin					Pectoral fin				Pelvic fin	
Lots	Vertebrae	Hyp.	Epur.	Upper rays	Lower rays	Pteryg.	Rays	Predorsal bones	Hard rays pteryg.	Hard rays	Soft rays pteryg.	Soft rays	Left side rays	Right side rays	Left side rad.	Right side rad.	Left side rays	Right side rays
WILD REFER.	24-25	3-5	3-4	8-9	7-8	11-14	13-16	2-3	9-11	10-12	11-15	12-16	13-17	15-17	4	4	6-7	6-7
INIT06	23-24			8-9	8-10		13-16			9-11		11-15	15-17					
LVIT01	24-25			8-9	8		14-15			10-12		12-13	15-16					
INIT07	23-25			8-9	8-9		13-16			9-14		11-14	7-16					
LVIT02	24-25			9	8-9		14-16			7-11		12-15	13-16					
INIT19	23-25	5-6	2-4	8-10	8-9	9-13	11-16	2-4	7-10	9-11	11-15	12-16	14-16	0-17	4	0-4	0-7	0-7
LVIT04	24	5	2-3	8-9	8-9	12-13	14-16	2-3	7-9	10-11	12-14	13-15	14-16	14-15	4	4	6-7	6-7
INIT18	23-25	4-5	2-4	9	8-9	11-13	14-17	2-3	7-9	9-11	11-15	12-16	14-16	14-16	4	3-4	6-7	6-7
LVIT05	24-25	4-5	2-4	9	8-9	11-13	14-16	2-3	7-9	9-11	12-15	13-15	14-16	14-16	4	3-6	6	6
INGR01	24-26	4-7	2-6	6-10	5-9	13-15	14-16	2-3	10-11	11-12	11-14	12-14	14-16	14-16	4	4	5-6	5-6
MEGR04	24-25	4-7	2-6	6-9	5-9	13-15	15-17	3-4	10-11	11	12-14	13-15	14-15	14-16	4	4	6	6

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

4% → 23 Vert. 23% → 25 Vert.

72% → 24 Vert. 1% → 26 Vert.

Results: Case 3

Skeletal anomalies

	Code	N. individuals	Frequency (%) of malformed individuals	Average anomalies load	Frequency of individuals with at least one severe anomaly (%)	Severe anomalies load	Frequency (%) of severe anomalies / total
Group 1	**INIT06**	55	**100**	**8.6**	**74.5**	**1.8**	16
	LVIT01	66	**100**	3.6	54.5	1.3	**19.5**
Group 2	**INIT07**	123	95.9	**3.8**	**52.8**	**1.6**	**23.2**
	LVIT02	122	**98.4**	2.7	48.4	1.2	21.2
Group3	**INIT19**	105	**96.2**	**5.8**	**47.6**	1.6	13.9
	LVIT04	40	92.5	3.9	17.9	**6.5**	**10.0**
	INIT18	105	86.7	**3.5**	**22.9**	**1.8**	**13.7**
	LVIT05	105	**96.2**	2.8	8.6	1.3	4.3
Group 4	**INGR01**	134	**95.5**	4.2	**28.4**	**1.5**	**10.5**
	MEGR04	126	93.6	**4.3**	27	1.3	9.1
Wild	**WIIT01**	72	54.8	1.4	4.2	2.3	21.2
	WIIT02	41	43.9	1.2	2.4	1	4.8
	WIIT03	60	55	1.6	1.7	3	5.8
	WIIT04	16	100	6.3	0	0	0
	WITU01	88	43.2	1.6	4.5	1.5	9.7

Skeletal anomalies

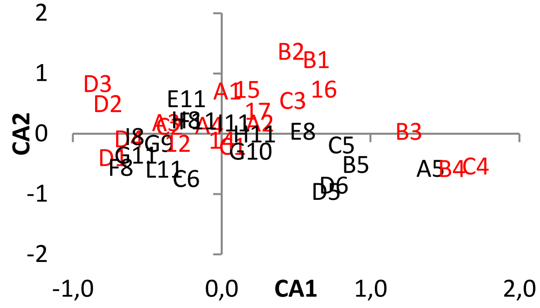
In order to understand why and how skeletal anomalies arise, the trend of meristic count variation and deformed individual rates was analyzed, taking into consideration the ossification typologies of the skeletal elements affected.

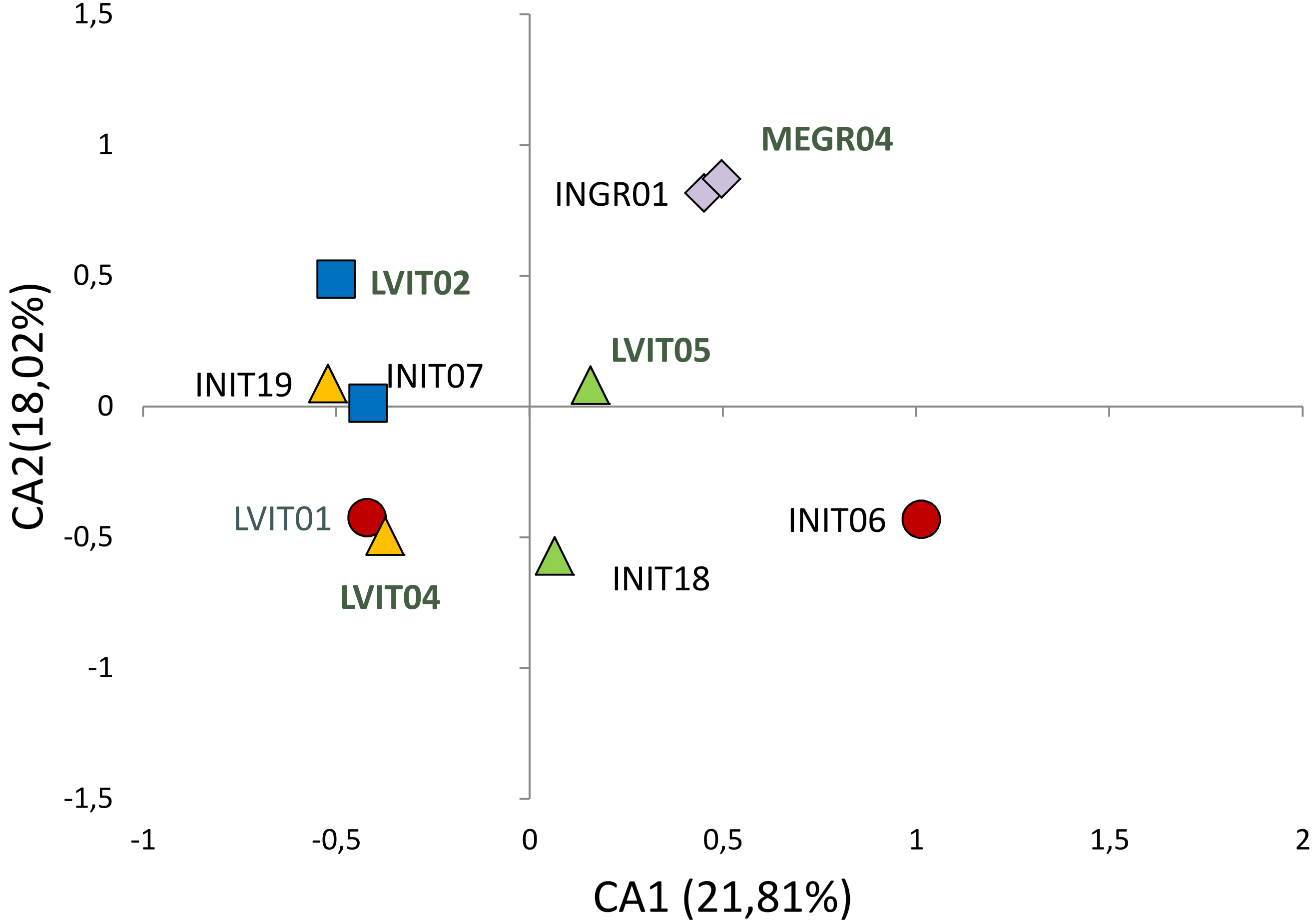
1. All the intramembranously ossified bones showed lower incidences of anomalies (and affected individuals) in semi-intensive rearing lots than in intensive one.

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 analisis: CA

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





The same indicator' colour specifies sister lots; semi-intensive lots are in green font

Results: Case 3

Quantitative analysis: NPMANOVA

***	= p≤0.001;
**	= p≤0.01;
*	= p≤0.05;
n.s. = not significant	

Group.1: INIT06-LVIT01***

Group 2: INIT07-LVIT02***

Group 3: INIT19-LVIT04 *; INIT18-LVIT05***; — Sisters lots

INIT19-INIT18***; LVIT04-LVIT05 n.s.; — Same origin – different age

Group 4: INGR01-MEGR04 ***

Differences are not significant only in LV lots sampled at differences ages

1. **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

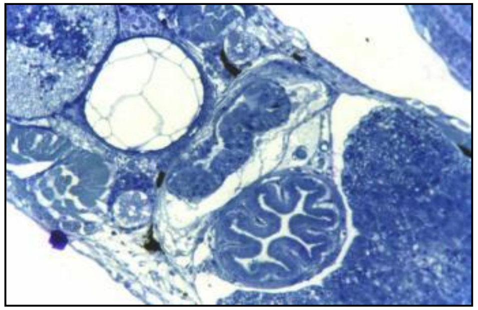
→ *semi-intensive rearing methodology seems to be a system at higher homeorhesis with 'canalised' developmental trajectories*

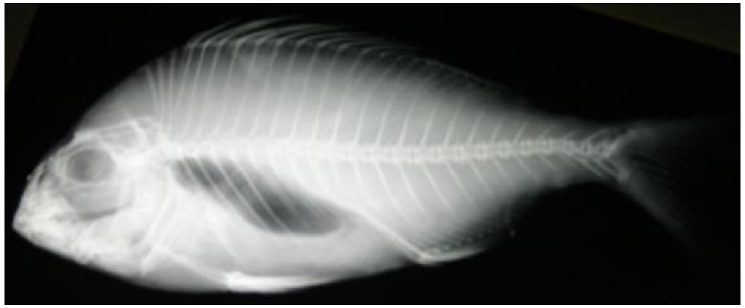
Materials and Methods: Case 4

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.

Study	Label	N	Characteristics
Case 4	SB	50 (17%)	presence of swim bladder
	noSB	237 (83%)	absence of swim bladder

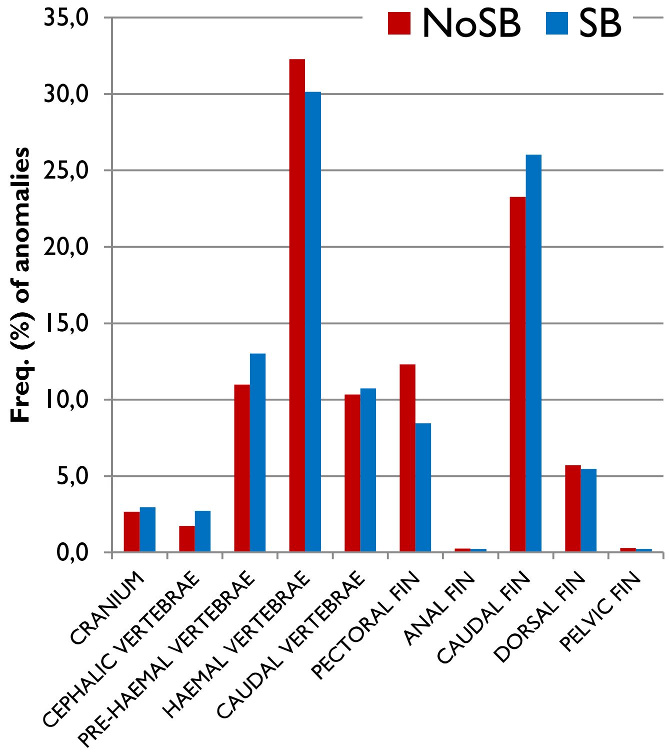




Skeletal anomalies

* Results obtained not considering the anomaly 12

	Code	N. individuals	Frequency (%) of malformed individuals	Average anomalies load	Frequency of individuals with at least one severe anomaly (%)	Severe anomalies load	Frequency (%) of severe anomalies
This study	SB	50	100.0	8.8	42.0	1.6	7.8
	NoSB	237	100.0	5.8	100.0	1.8	17.2
	NoSB*	237	100.0	9.7	48.5	1.7	8.7



- ✓ 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*

Conclusions

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 m^3) 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!