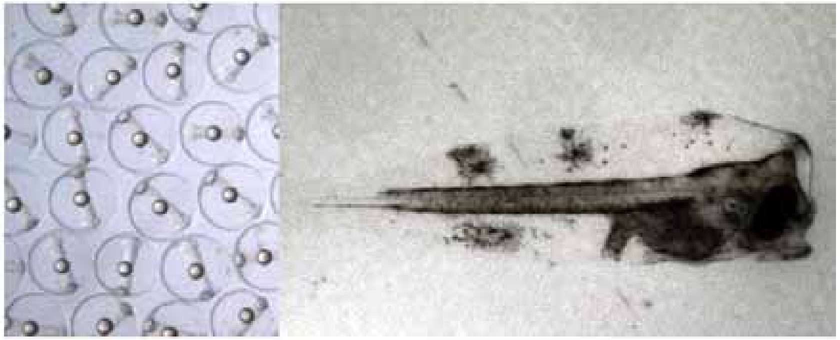
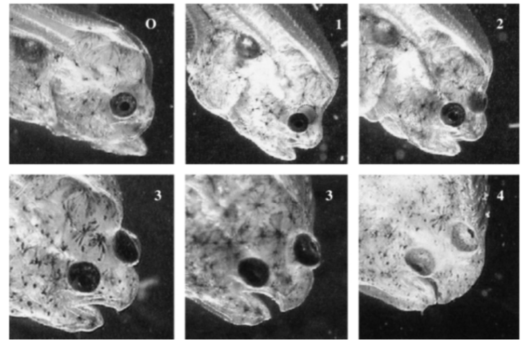
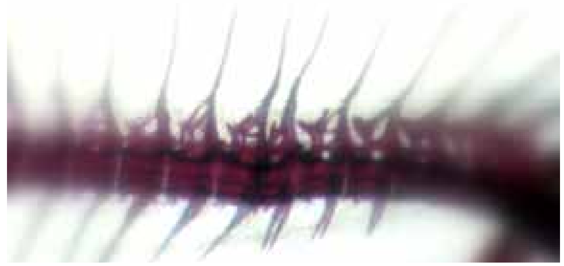




New insights of the effects of dietary vitamin A on flatfish skeletogenesis: the case of *Solea senegalensis*



Ignacio Fernández and Enric Gisbert

IRTA RECERCA I TECNOLOGIA AGROALIMENTÀRIES

Fish Nutrition and Larval Rearing Group

IRTA – Sant Carles de la Ràpita; Spain

Larvi’09. Ghent, September 2009

Main flatfish world aquaculture production

World aquaculture production	in Tones	in Thousands US $
Flatfishes	7%	12%
Total marine fish	1737,080	5884,840

Production increase (2000-06)	Production	Value in Thousands US $
Europe	100%	100%
World	400%	100%

fao.org

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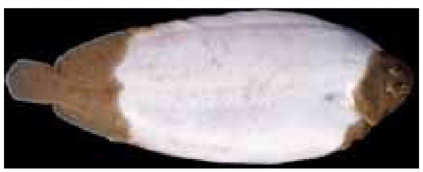
Flatfish aquaculture production problems



- Low quality and discontinuous spawnings — Howell et al., 2009
- Mortality by pathogen infection — Zarza et al., 2003
- Pigmentary disorders — Hamre et al., 2007
- High incidence of skeletal deformities and abnormal eye migration (40-90%) — Aritaki et al., 1996; Takeuchi et al., 1998; Gavaia et al., 2002; Lewis and Lall, 2006; Fernández et al., 2009

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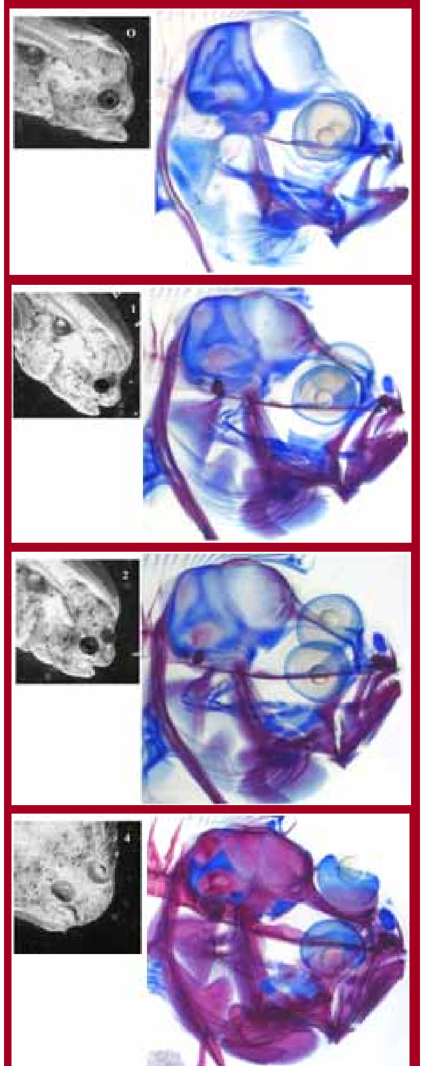
Flatfish larvae development



From Fernandez-Diaz et al., 2001 and Schreiber 2006.

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**

Flatfish larvae development species-characteristics

General patterns of metamorphosis:

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

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

Fish species	Hatch	First feeding (PH)	Metamorph start (PH)	Metamorph End (PH)
Japanse flounder	63h 40ºC day	4days 60ºC day	12-14days 210ºC day	40days 525ºC day
Summer flounder	48-60h 36-45ºC day	23 days 425 ºC day	35 days 647ºC day	65 days 1170ºC day
Atlantic halibut	15 days 70ºC day	<50 days 265ºC day	81-90 days 1035ºC day	135 days 1552ºC day
Senegal sole	24-48h 34ºC day	3 days 51ºC day	10 days 170ºC day	20 days 340ºC day

Osse and Van der Boogaart, 1997

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).

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Different factors that affect bone development

Xenobiotic Factors	Abiotic factors	Biotic factors
	• Light	
• Contaminants	• pH	• Culture density
• Pesticides	• CO_2, O_2	• Pathogens
• Herbicides	• Temperature	• Genetics
• Heavy metals	• Radiation	• Nutrition
• Organochlorates	• Salinity	
	• Water flow	
	• Tank characteristics	

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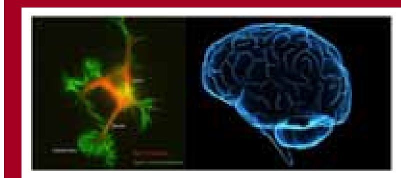
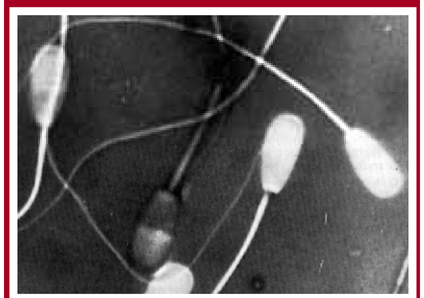
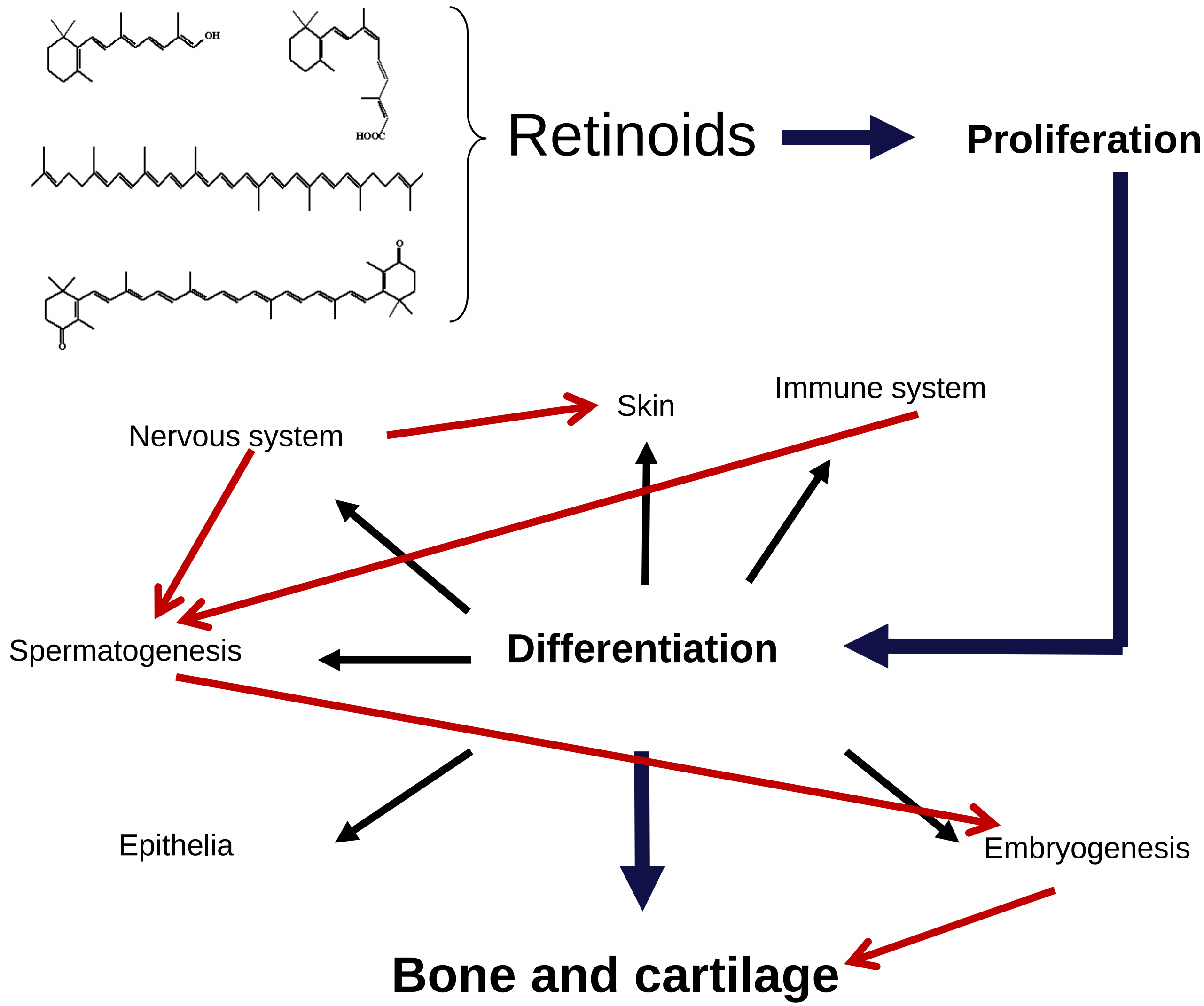




Vitamin A roles during development



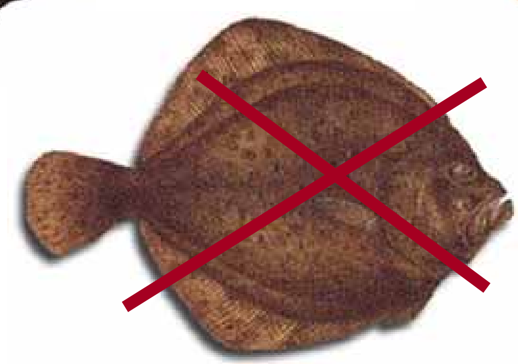
Ross et al., 2000

Objective

Dietary VA effects on skeletogenesis:

1. **Broodstock spawning performance and quality**
2. **Juveniles skeletogenesis**
3. **Larval performance and 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.



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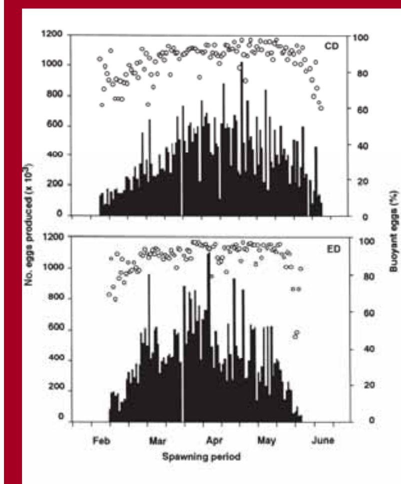


	Control diet (n=10)	Experimental diet (n=10)	Caught in wild†
Retinol	1.35±0.70	5.42±1.47*	0.30±0.03
Retinal	6.30±0.54	8.21±0.82*	10.54±1.50
Total vitamin A	7.66±0.48	13.63±1.41*	10.84±1.48

* Experimental diet is significantly different from the control diet in the same row (P<0.005).
† Unfertilized eggs (n=5).

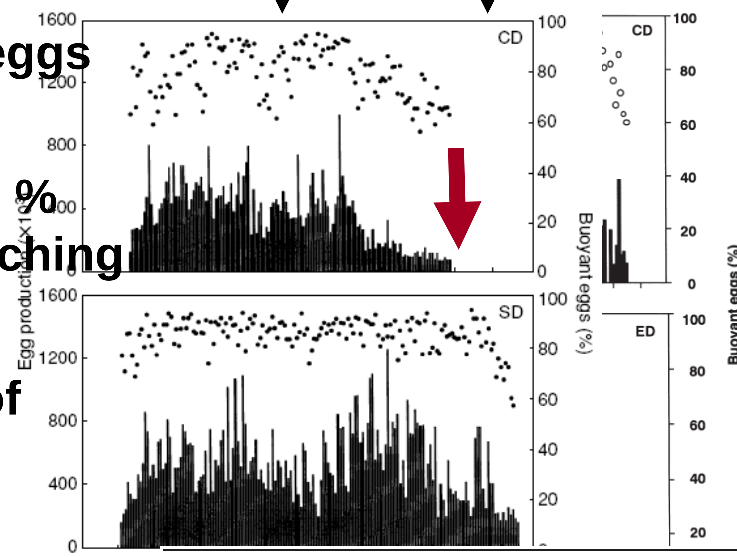
Japanese flounder broodstock-spawned egg quality

	NSD	CD	SD	ED
Dietary VA (IU kg^{-1})	2,566	11,000	56,333	337,000

Retinyl acetate

Retinyl palmitate

- •NSD: feed intake decreased and stopped spawning
- •CD: longer spawning period and higher nº eggs spawned
- •SD: Higher egg production, % buoyant eggs, but no differences in hatching rate
- •ED: Higher fecundity, % buoyant eggs and hatching rate
- •No differences in % of abnormal larvae and survival rate
- •SD: lower % of abnormal larvae
- •No differences in egg VA content
- •ED: Ovaries and eggs higher retinoid content, being retinol the most abundant contrary to wild and CD eggs where it was retinal



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† Unfertilized eggs (n=5).

Furuita et al., 2001; 2003

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Atlantic halibut-juveniles

	Diet				
	Control	Low Phosphorus	Low Vitamin C	High Vitamin A	Oxidized Oil
Liver lipid (%)[2]	11.22±0.60[a]	11.27±1.14[a]	7.04±0.84[b]	11.63±0.90[a]	12.18±1.20[a]
Liver retinol (μg g[-1] liver)[9]	5.60±0.81[a]	5.17±0.22[a]	4.06±0.67[a]	11.37±0.73[b]	5.58±0.17[a]

	Control (n=16)	High Vitamin A (n=16)
No. cephalic vertebrae	3.86±0.10	3.79±0.11
No. prehemal vertebrae	11.86±0.10	12.14±0.10
No. hemal vertebrae	31.14±0.23	31.29±0.22
No. caudal vertebrae	3.86±0.10	3.57±0.14
No. total vertebrae	50.71 ±0.34	50.79±0.24

[1]Mean ± standard error

B: scoliosis
C: vertebral fusion
D: vertebral compression

	Control	High VA

Diet				
Control	Low	Low Vitamin	High	Oxidized Oil

	Diet														
	Control (n=10)			Low Phosphorus (n=10)			Low Vitamin C (n=10)			High Vitamin A (n=10)			Oxidized oil (n=10)		
Abnormality	No.	FA	No. of Fish	No.	FA	No. of Fish	No.	FA	No. of Fish	No.	FA	No. of Fish	No.	FA	No. of Fish
A.	0	0	0	3	0.67	1	0	0	0	0	0	0	12	8.63	1
B.	0	0	0	63	13.97	5	25	59.52	3	79	92.94	6	120	86.33	7
C.	0	0	0	0	0	0	4	9.52	2	2	2.35	1	3	2.16	1
D.	0	0	0	0	0	0	4	9.52	1	4	4.71	2	0	0	0
E.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F.	0	0	0	197	43.68	5	0	0	0	0	0	0	0	0	0
G.	3	100	2	2	0.44	2	5	11.90	4	0	0	0	3	2.16	3
H.	0	0	0	0	0	0	4	9.52	3	0	0	0	1	0.72	1
I.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
J.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L.	0	0	0	186	41.24	5	0	0	0	0	0	0	0	0	0
M.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
O.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Q.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total abnormalities	3			451			42			85			139		
Vertebral element abnormality	3	100		66	14.63		33	78.57		85	100		135	97.12	
Neural element abnormality	0	0		199	44.12		9	21.43		0	0		4	2.88	
Hemal element abnormality	0	0		186	41.24		0	0		0	0		0	0	

[1] FA was determined by dividing the number of times each abnormality was observed by the sum of the total abnormalities observed and multiplied by 100

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

Lewis and Lall, 2009 (sub to J. Appl. Ichthy.)

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Japanese flounder-larvae

Two different approaches

Balneation

•*Hoxd-4* expression was affected by > 0.1 μM RA treatment in a dose response manner

•Implied pharyngeal arches development

(Suzuki et al., 1998).

•RA treatment depressed *hoxd-4* and *shh* expression

•malformed pharyngeal and trabecular cartilages

(Suzuki et al., 1999).

•RA and disufiram treatment affected to pharyngeal and pectoral skeleton

(Suzuki et al., 2000).

•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).

•6-9dph larvae exposed to RAR- and RXR selective agonist (25nM) presented jaw deformities

(Haga et al., 2003).

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Japanese flounder-larvae

Two different approaches

Balneation

Feeding → Live prey enrichment with VA

Larvae fed atRA

Enriched VA live prey

VA palmitate

•Artemia metabolized atRA to another retinoids,but no isomerized RA

•Larvae isomerized RA

(Haga et al., 2004).

•Safe level (flounder skeletogenesis) of VA in Artemia less than 50,000 IU kg^{-1}

(Dedi et al., 1995).

•Larvae (18-21dph): max. accumulation of atRA at 3h post feeding, at 18h decreased in 50%

•When enriched Artemia with 200*10^{6} IU kg^{-1} of different VA compounds, all fishes presented compressed vertebrae

(Takeuchi et al., 1998).

•Rotifer: higher VA content when enriched in darkness

•Artemia: VA con[...] light conditions

•Artemia VA con[...] enrichment

(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).

•F-G larvae (27-31dph) fed Artemia enriched increasing VA palmitate levels induced hypermelanosis and vertebral deformities

(Tarui et al., 2006)

Retinyl acetate

•Rotifers accumulated more efficiently VA compounds

(Giménez et al., 2007).

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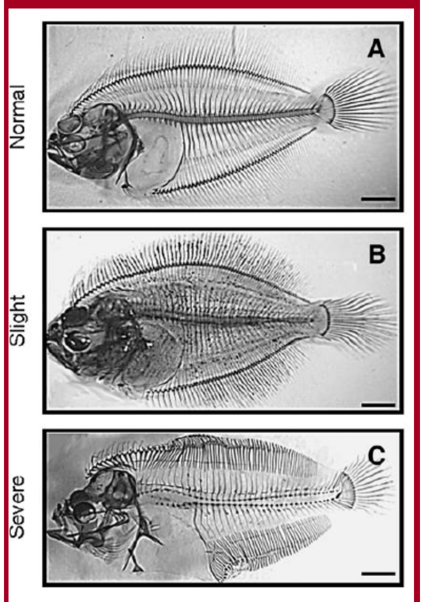
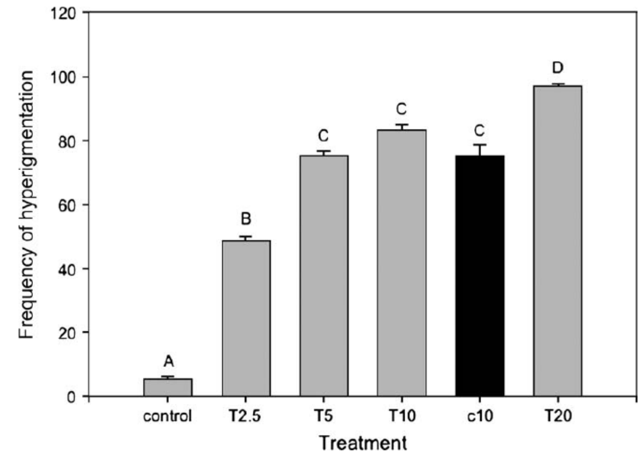
Summer flounder- pre-metamorphic larvae

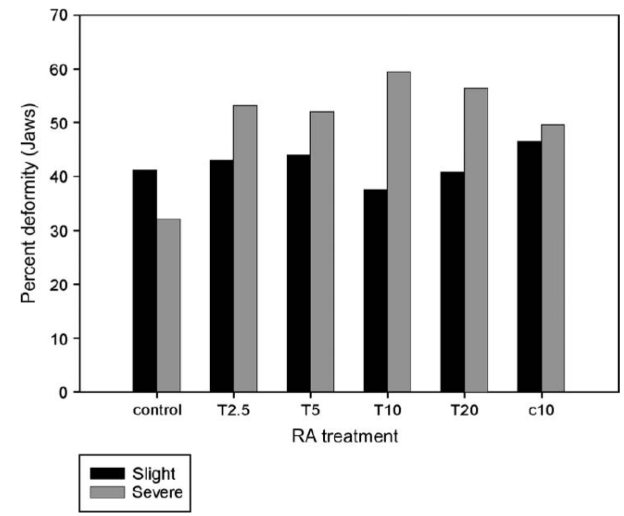
Balneation	atRA	atRA	atRA	atRA	atRA	9-*cis*-RA
nM	0	2.5	5	10	20	20

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

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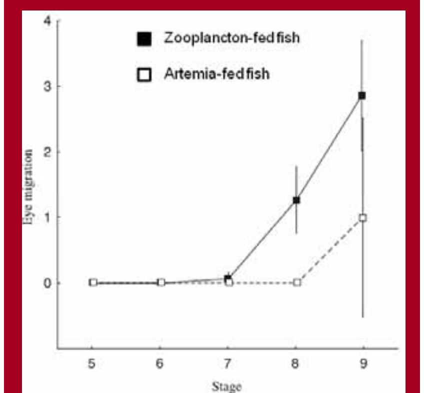
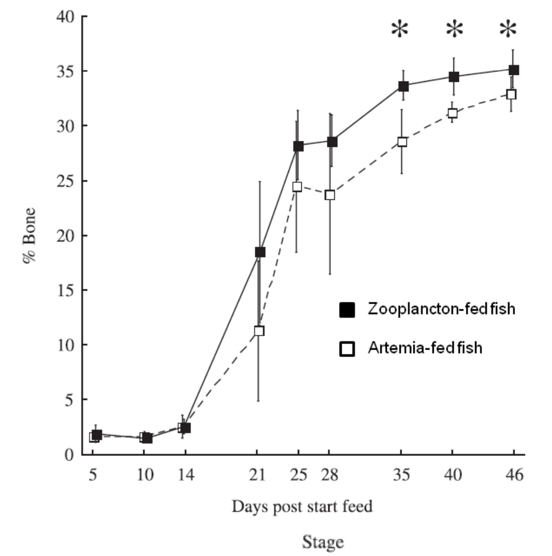
Atlantic halibut- larvae

	Enriched artemia	Zooplankton
Dietary VA content	466 IU kg^{-1}	66 IU kg^{-1}

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.

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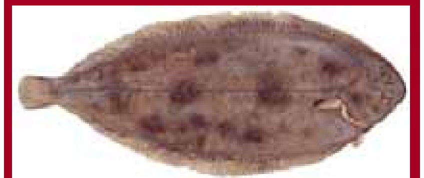
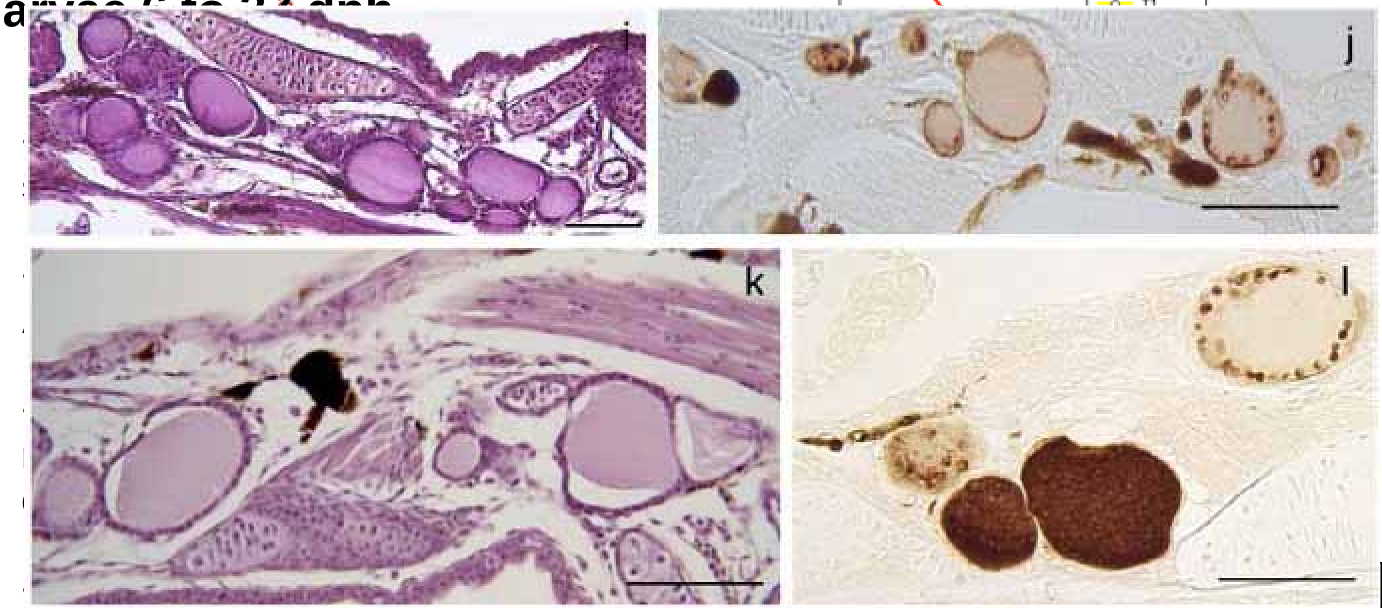
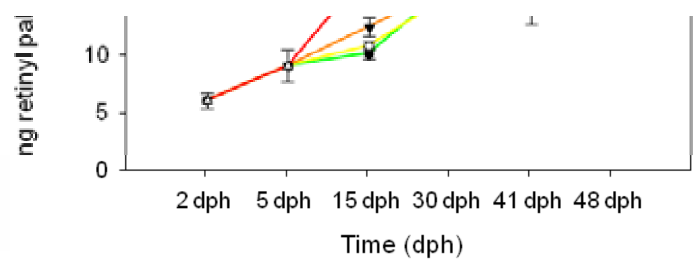


Senegalese sole- larvae

(Retinyl palmitate)	Control	T1	T2	T3
Artemia VA content (IU kg^{-1})	37,000	44,666	82, 666	203,000

31 days feeding (Retinyl palmitate)
larvae 6 to 37 dph





eye migration at 10 dph

•Lower nº of thyroid follicles, but higher size and T_3 and T_4 immunoreactivity with higher VA dietary content at 48 dph

Fernández et al., 2009.

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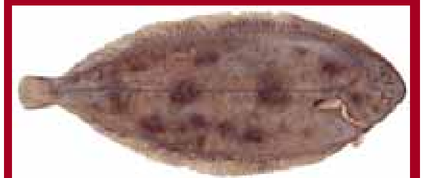
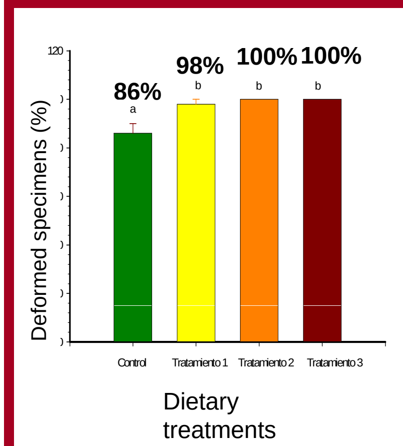




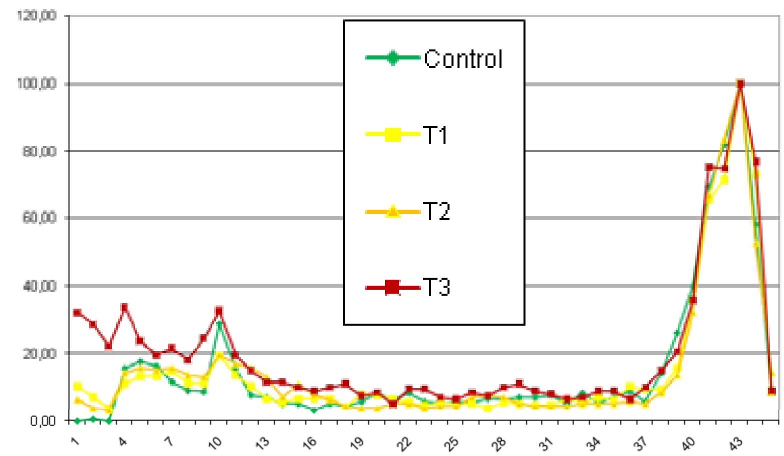
Senegalese sole- larvae

	Control	T1	T2	T3
Artemia VA content (IU kg^{-1})	37,000	44,666	82, 666	203,000

31 days feeding

larvae 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**



Fernández et al., 2009.

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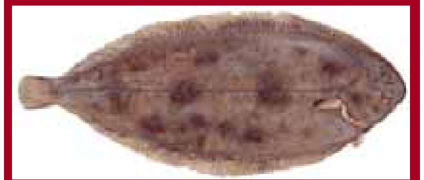
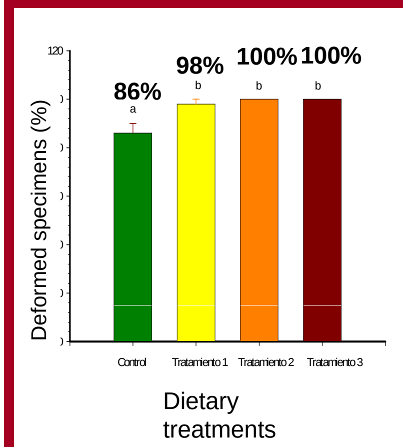
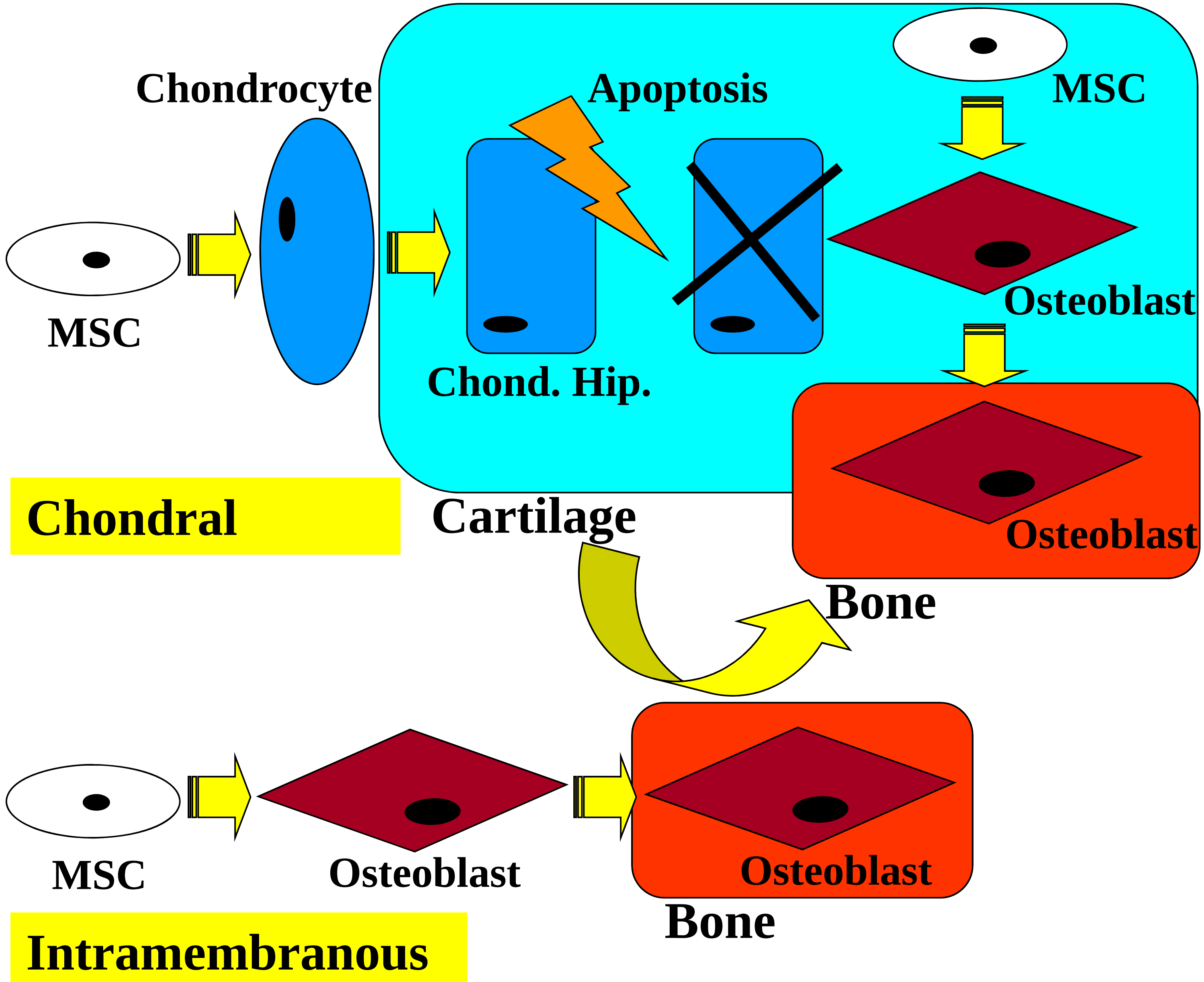
Senegalese sole-larvae







Fernández et al., 2009 (sub to J. Appl. Ichthy.)

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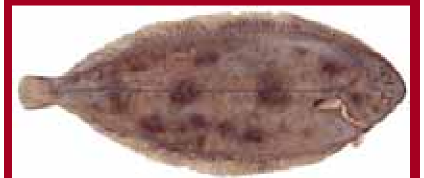
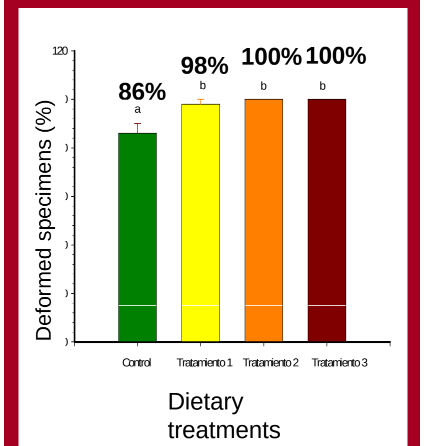
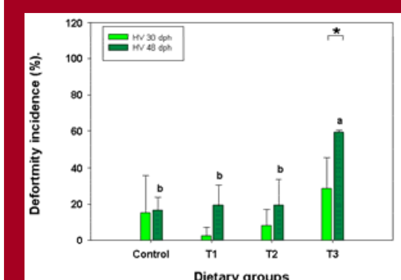
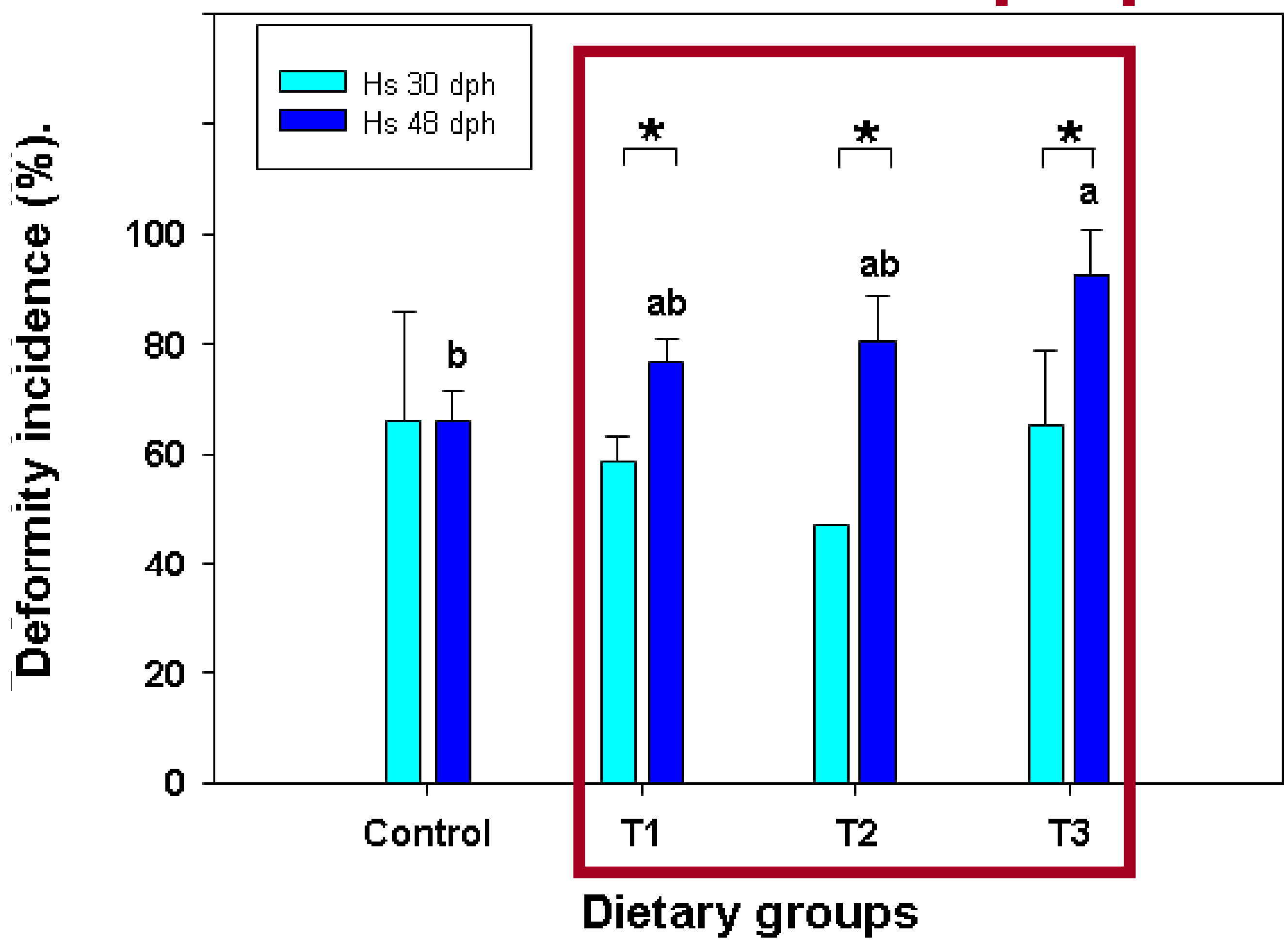
Senegalese sole-larvae

	Control	T1	T2	T3









•Bone ossification: different sensitivity to VA dietary imbalance

Fernández et al., 2009 (sub to J. Appl. Ichthy.)

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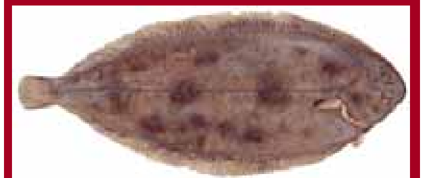

Senegalese sole-larvae

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

	Development larvae stage			
Dietary treatments	Pre-metamorph (3-10 dph) Rotifer	Pro-metamorph (10-20 dph) Artemia	Post-metamorph (21-37 dph) Artemia	Weaning (33-55 dph)
T1	Easy selco (ES)			Dry feed
T2	Easy Super Selco			
T3	ES+ VA(x10)	ES		
T4	ES+ VA(x50)	ES		
T5	ES	ES+ VA(x10)	ES	
T6	ES	ES+ VA(x50)	ES	
T7	ES		ES+ VA(x10)	
T8	ES		ES+ VA(x50)	

Fernández et al., 2009 (in prep.)

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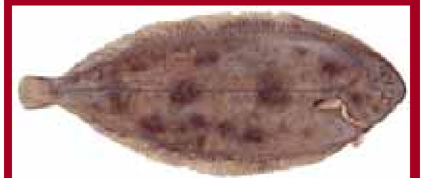
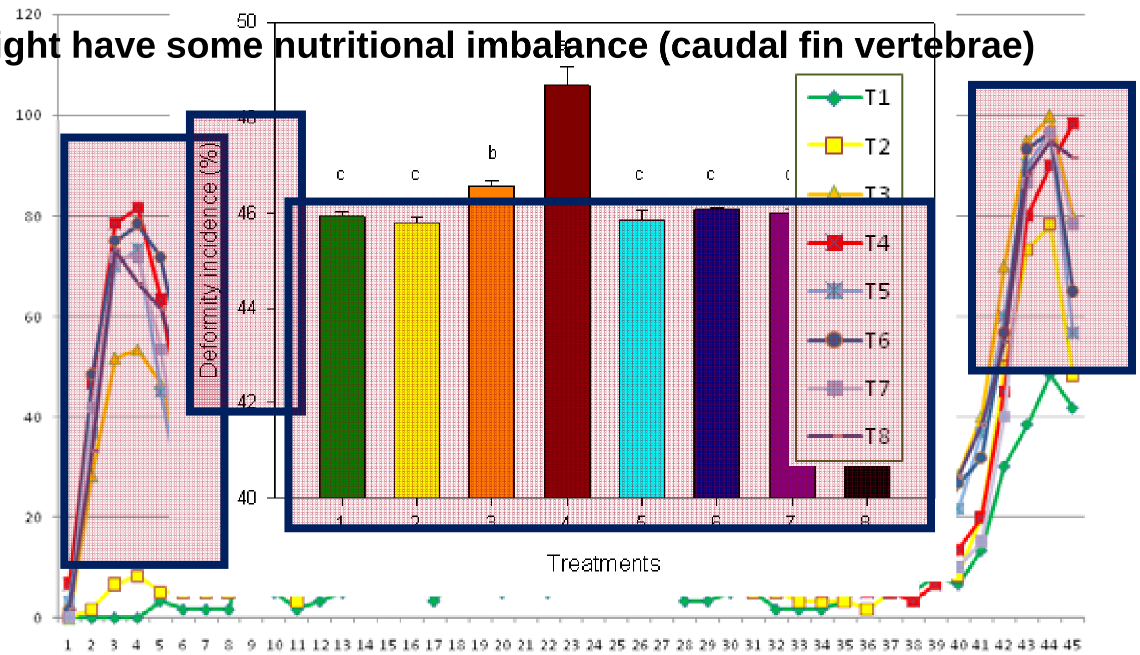


Senegalese sole- larvae

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.)

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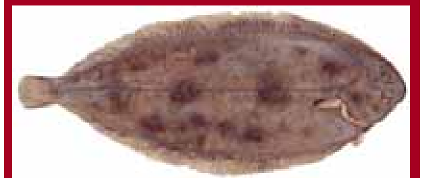

Conclusions: Senegal sole

- 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

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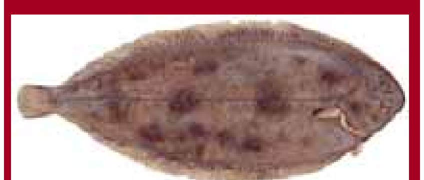

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Conclusions: Flatfishes

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**

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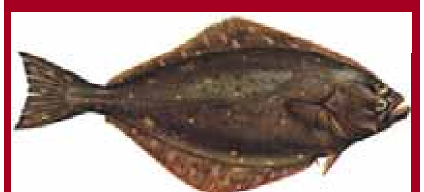

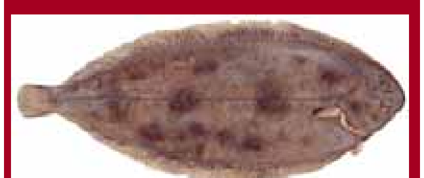

Conclusions:

9. **VA could reduce pigmentary 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 (ºc 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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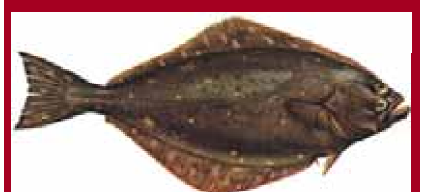
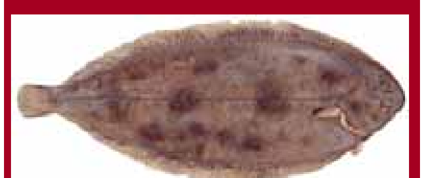
Acknowledgements :





Thank you for your attention!!!

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