Dietary vitamins as a causal factor for malformations in seabass and seabream.

A review of current knowledge with recommendations for practical application

Ifremer

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Why the vitamins?

- Marine fish larvae feeding sequence contained significant amounts of lipophile vitamins like vit. A and D. Besides their strict nutritional role, these vitamins can have physiological effects
  - Vit A: involved in night vision, antioxidant, associated with cell differentiation, controls the expressions of many genes involved in morphogenesis (can be teratogenic)
  - Vit D is an hormone that maintain Ca homeostasis and directly acts on bone cells.

- Other vitamins, hydrophiles, are also largely used/found in marine fish feeding sequence…
  - Vit. C: antioxydant, essential for collagen synthesis, and participates to several metabolic processes.

The experimental approach…

- Sea bass larvae:
  - Larvae were exclusively fed with compound diets from mouth opening
  - The control diet incorporated the vitamin mix 762 recommended by NRC93
  - Vitamin mix normally represented 8% of the formula
    - We made a 2X vitamin mix for more flexibility
    - 4% 2X= 8% 1X
- Sea bream larvae:
  - Experiments were based on live prey enrichments

Sea bass larvae

VITAMIN MIX

Rationale

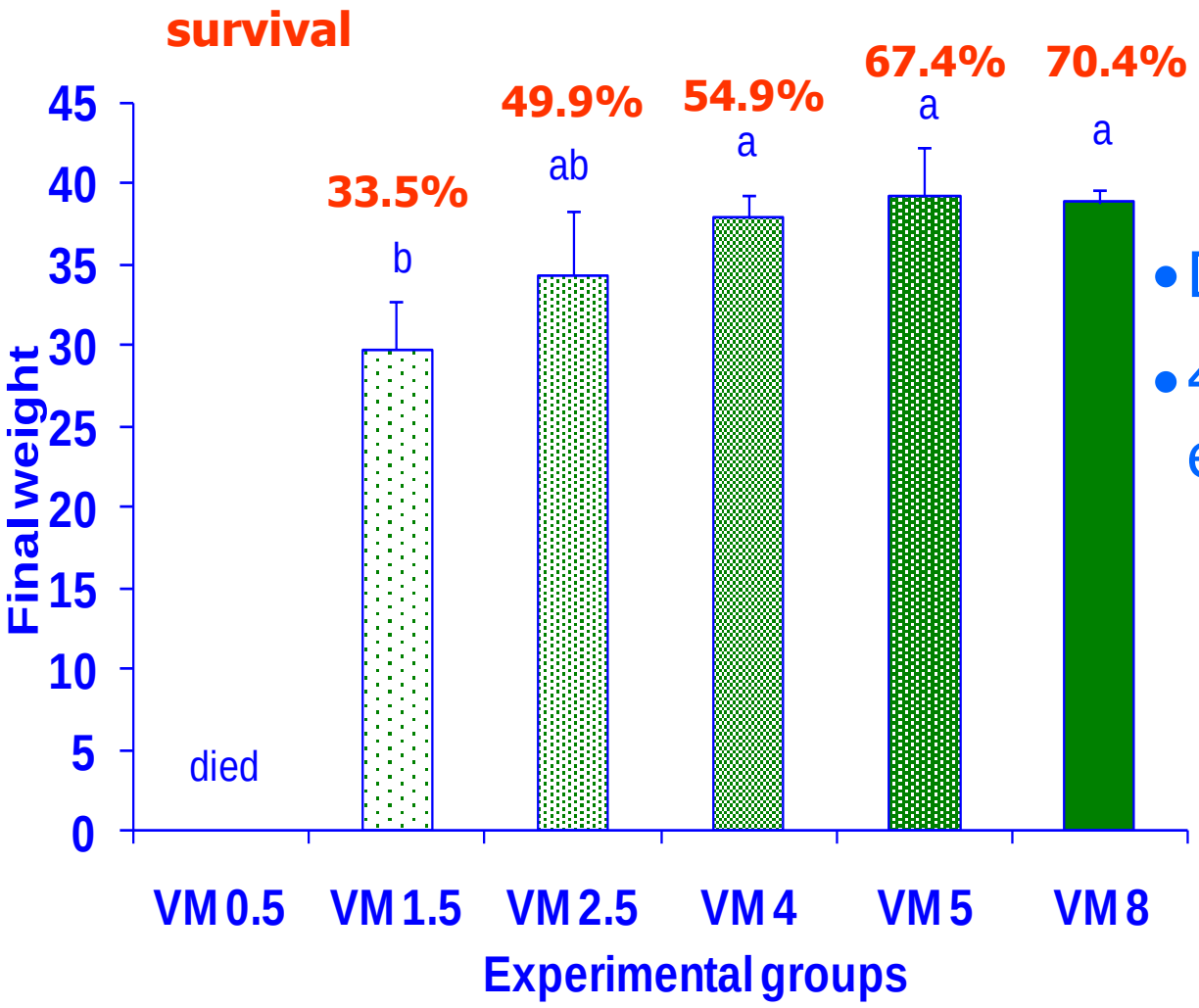
Vitamin mix incorporated in larvae diets = 8 times vitamin mix 762 (NRC 93 recommendations, established for juveniles)

➔ is it appropriate?

Experimental design

- 6 Vit Mix levels: 0.5, 1.5, 2.5, 4, 5, 8 %
- Same batch of defatted fish meal for all coming experiments:
  - Avoid uncontrolled vitamin supplies
  - Incorporation of marine PL for adjusting HUFA level
- 3 replicates per experimental group
- Growth and survival
- Gene data: accessing possible influence on genes controlling morphogenesis
- Observation of larvae morphogenesis at day 38 (staining of cartilage and bone: alcian blue & alizarin red)

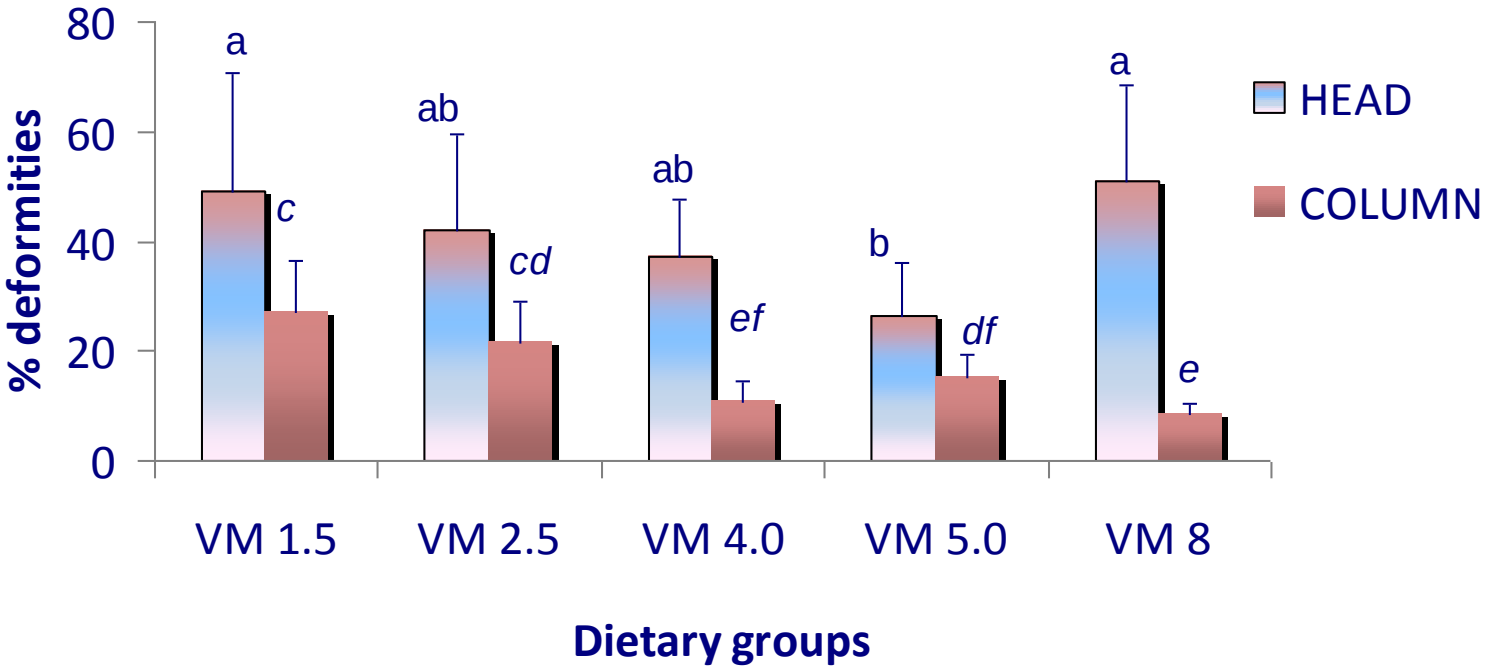
Larvae weight at d38



- Diets are very efficient
- 4 % kept for the next experiments

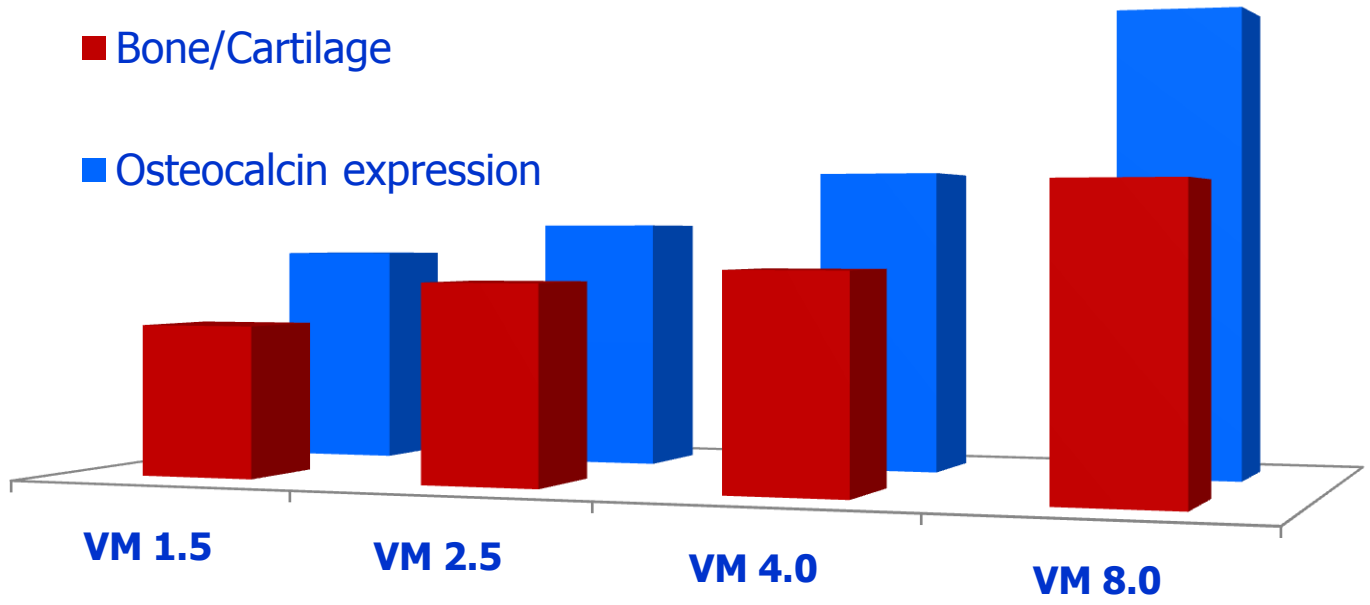
Percentages of head and column deformities

- "Worst" results with 1.5% and 2.5%
- "Best" results with 4%-5%
- Important % of deformities: necessary to refine the proportion of some vitamins

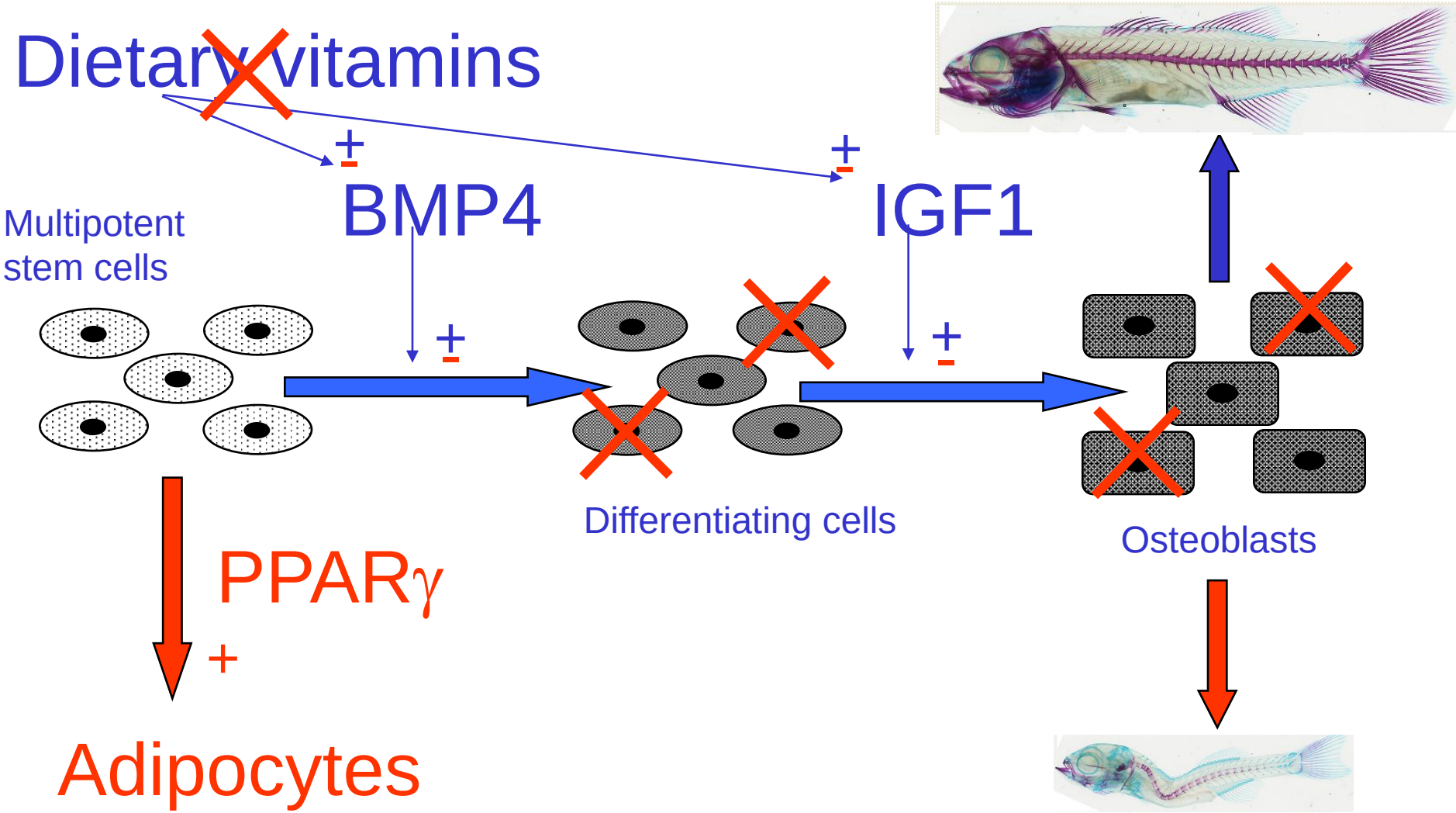


Ossification status in 38 day-old larvae

- The higher the % of VM, the faster the ossification process
- Osteocalcin expression well correlated with ossification



Effect of dietary vitamins on the ossification process in sea bass larvae



Conclusions and recommendations

(Vitamin mix experiment)

- Necessary to refine the proportions of certain vitamins in the vitamin mix VM 4.0
- Vitamin mix level in diet may alter osteoblast differentiation by disrupting a temporal sequence of coordinated gene expression
- Involvement of PPARγ: we should not stimulate by diet its expression during early larval stages i.e. *avoid incorporation of high HUFA levels in larvae diets during the first two weeks of life*

Sea bass and Sea bream larvae

VITAMIN A

Importance of Vitamin A

- **Vitamin A:** *involved in night vision, antioxidant, associated with cell differentiation (immune system and the reproduction and growth processes), controls the expression of many genes involved in morphogenesis (can be teratogenic)*

- **Does the dietary vitamin A level appropriate?**
  - *In the vitamin mix?*
  - *In the enrichments?*

SEA BASS EXPERIMENTS

VITAMIN A

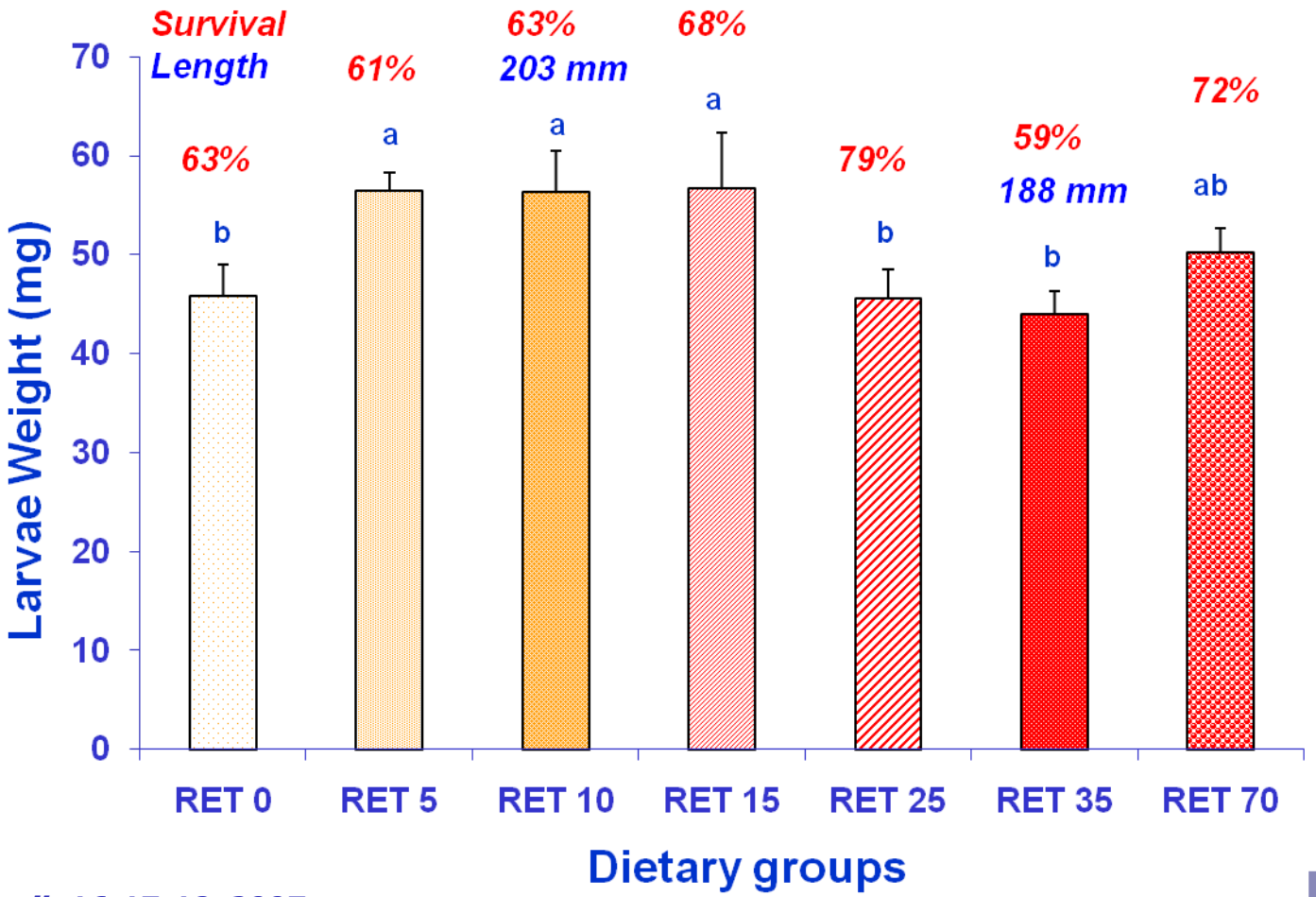
Experimental design

for sea bass larvae

- 7 vitamin A (*retinol acetate*) levels tested:
  - 0, 5, 10, 15, 25, 35, 70 mg/Kg of diet
    - *(called RET 0, RET 5 …)*
  - 35mg/kg ~120000 IU/kg diet
- 3 replicates per experimental group
- Duration: 45 days, and animals kept up to 2 g.
  - Growth and survival
  - Investigation of ossification process and deformities

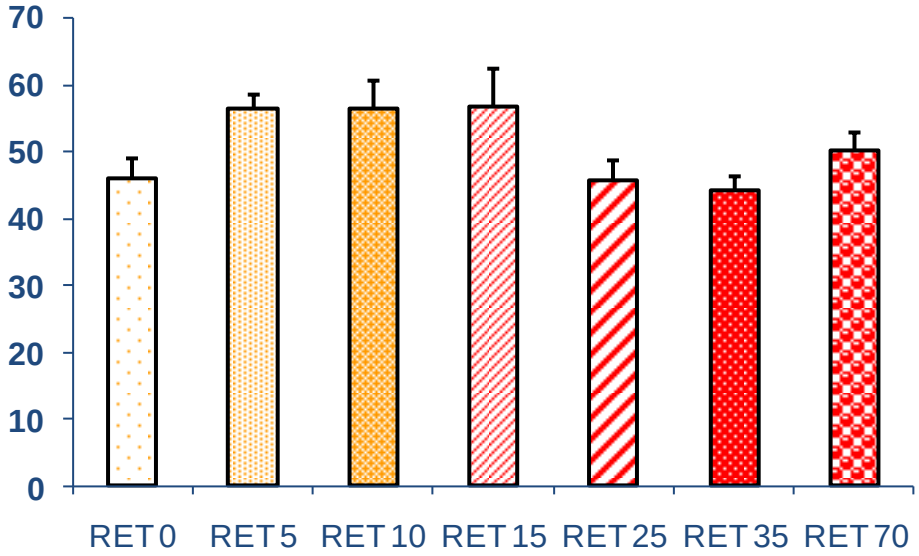
Growth and data survival at day 45

- Very good zootechnical results
- Best results obtained with VIT A levels < 35 mg/kg (normally recommended)

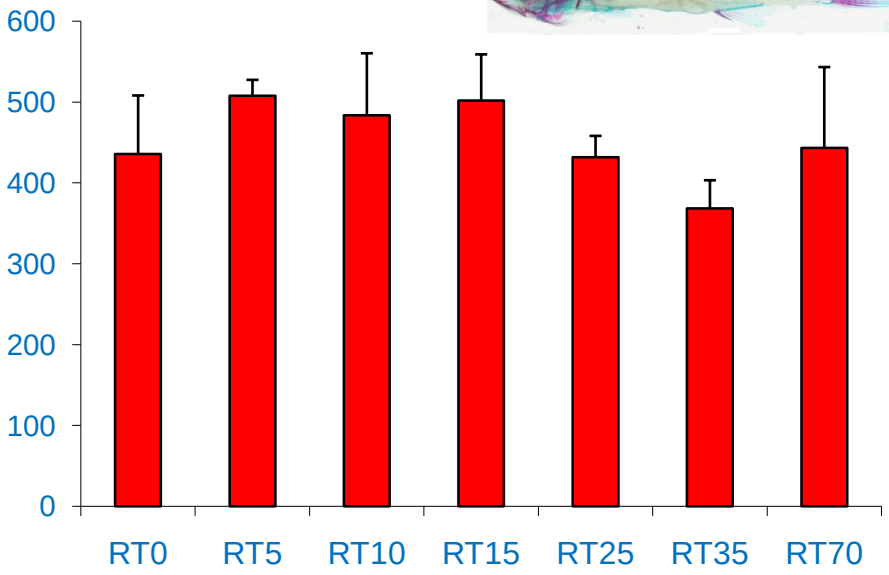


Ossification at day 45

- Growth: best for levels RET 5 ➔ RET 15
- High correlation with ossification level (r= 0.92)

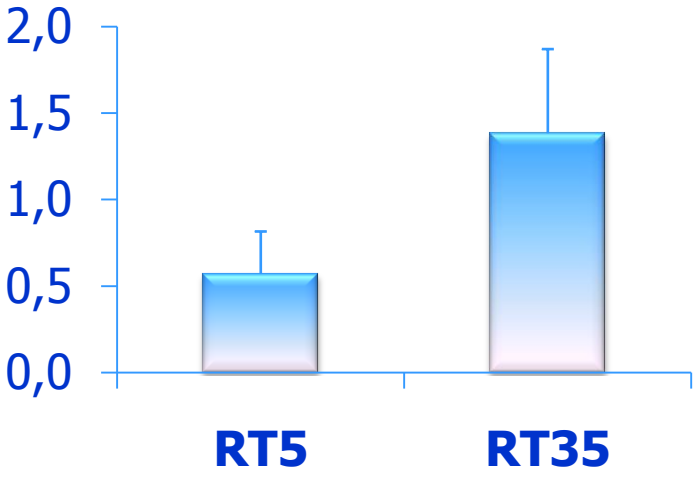


Growth in mg



Surface of mineralized bones/larvae

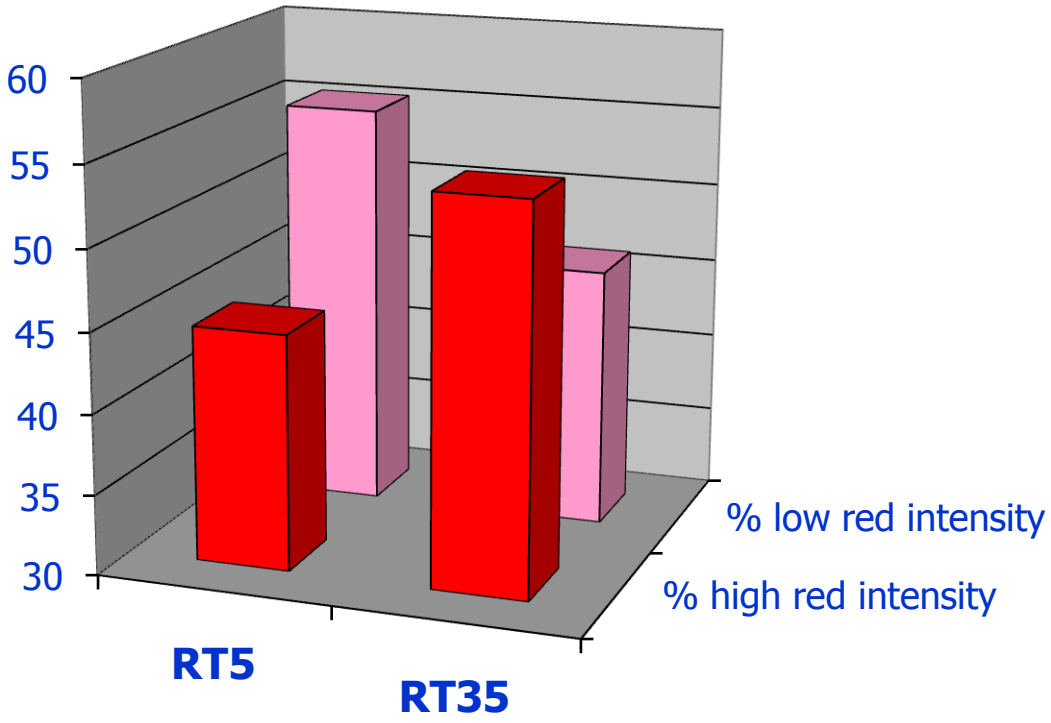
Ossification



Osteocalcin expression

Marker of bone cell

Red intensity obtained by staining

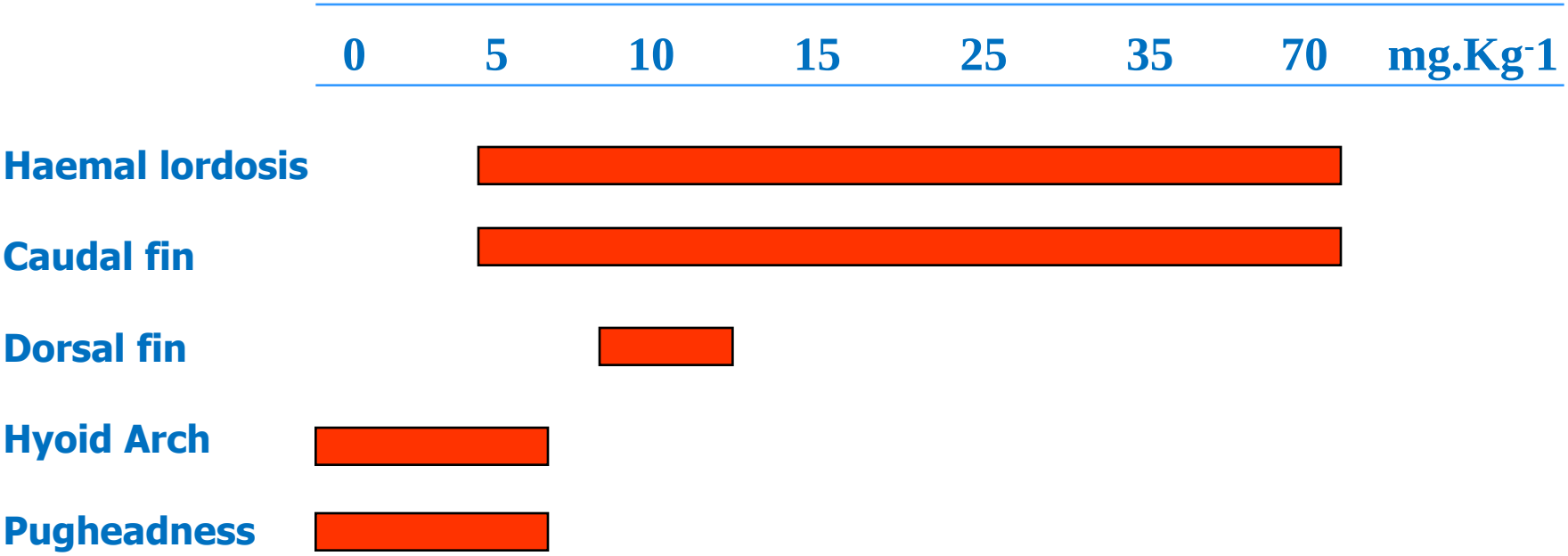


☞There is likely a regulation of ossification/mineralization process by retinol

Deformities

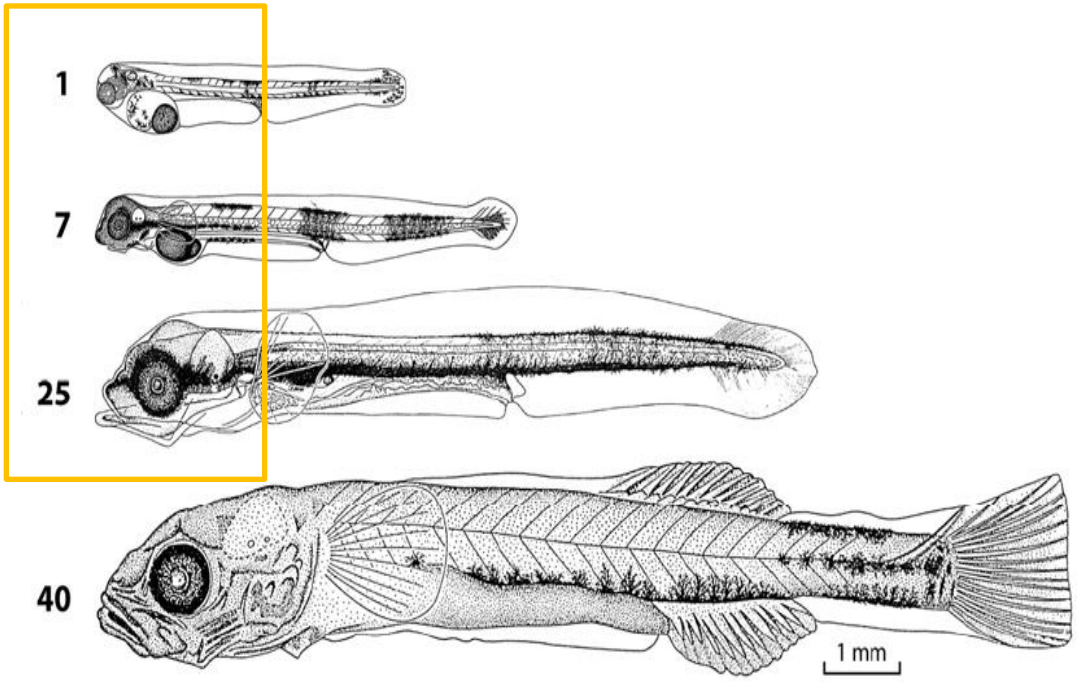
*Analyses performed by **Giorgos Koumoundouros***

- Optimum levels of dietary retinol for reduced deformities incidence depend on malformation type



Deformities

- The structures that develop earlier were less affected by the lowest dietary vitamin A levels
- Head area mainly develop during the 3 first weeks post-hatching



Skeletal structures development

dph

☞ **adapt dietary vit A level to the larval stage**

Preliminary conclusions-1
(sea bass experiment)

- High dietary vitamin A levels affects larval growth
  - best growth for RET5 ➔ RET15
  - *Actual levels are probably too high*
- Vitamin A may likely regulate ossification and mineralization
- Optimum levels of dietary retinol for reduced deformities incidence depend on malformation type
  - Dynamic nutrition? Should adapt the level to the developmental stage…

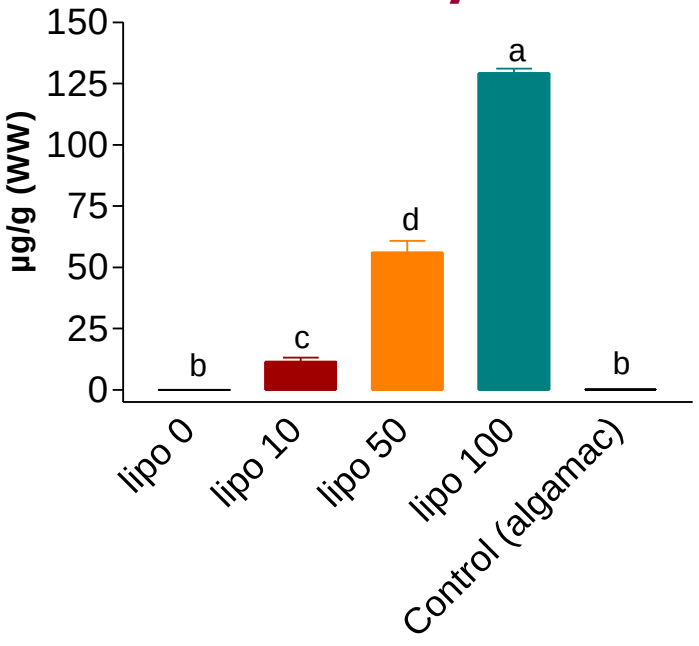
SEA BREAM EXPERIMENTS

Development of a reliable Vitamin A enrichment procedure for live feeds

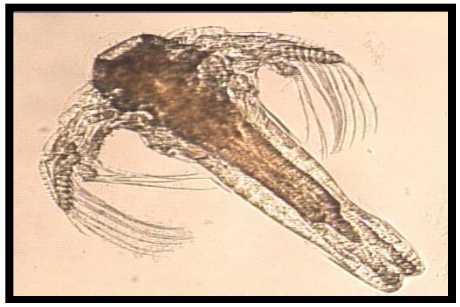
Vitamin A dosage effect on skeletal deformity in seabream larvae at two time windows

VITAMIN A

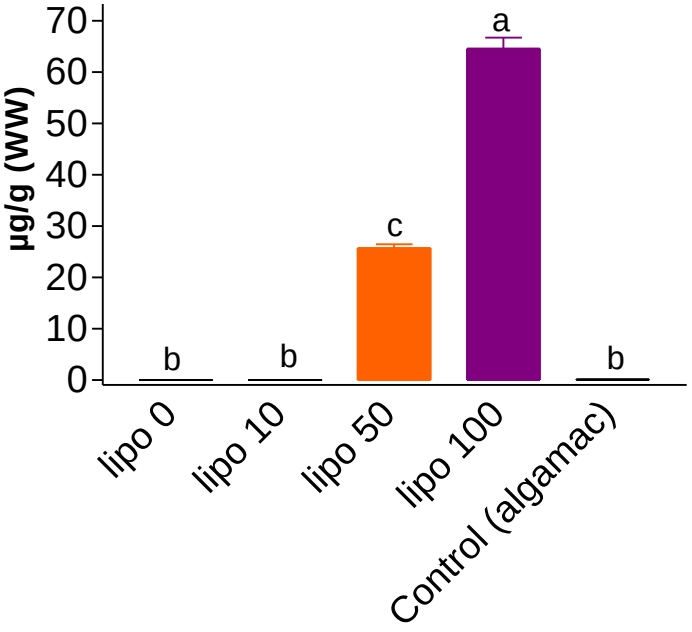
Rotifer – retinol + retinyl acetate



Treatment (Apparent Vit A dose)



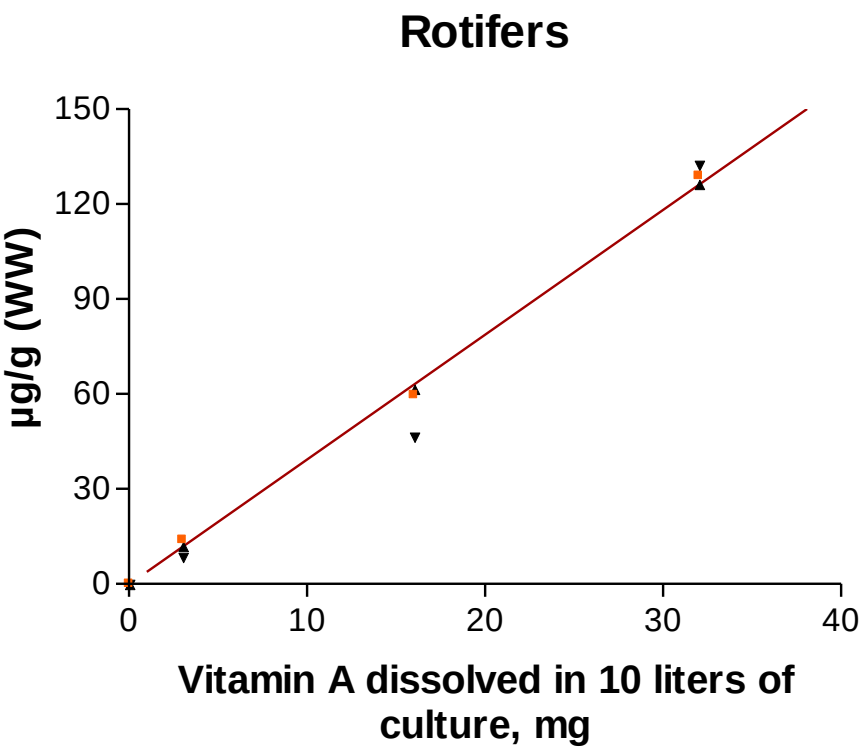
Artemia - retinol + retinyl acetate

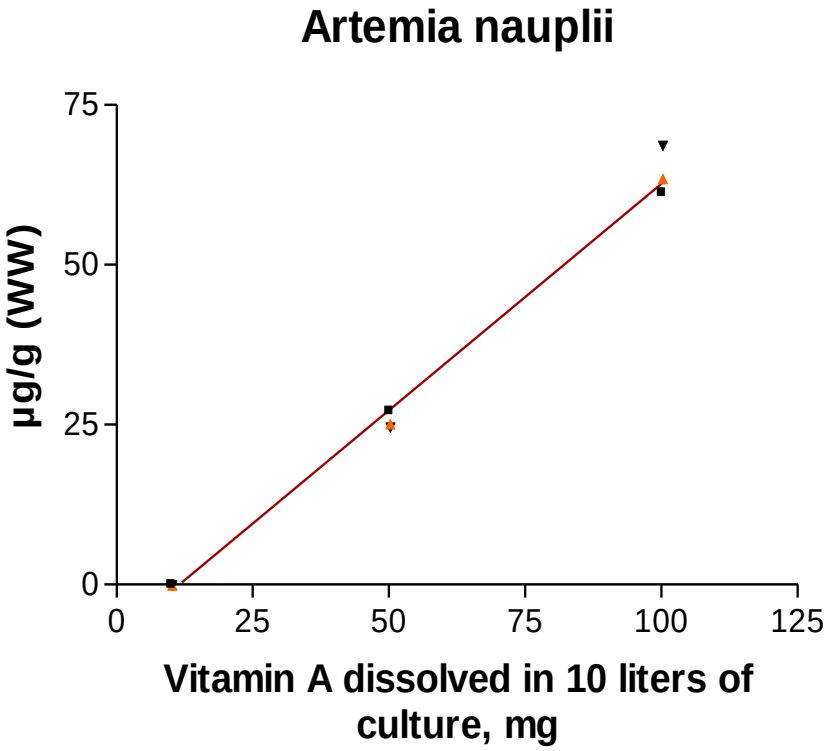


Treatment (Apparent Vit A dose)

Linear regression

Total vitamin A content in LP increased linearly with increasing levels of retinol acetate into the enrichments





Vitamin A dosage effect on skeletal deformity in seabream larvae at two time windows

Experimental design

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34

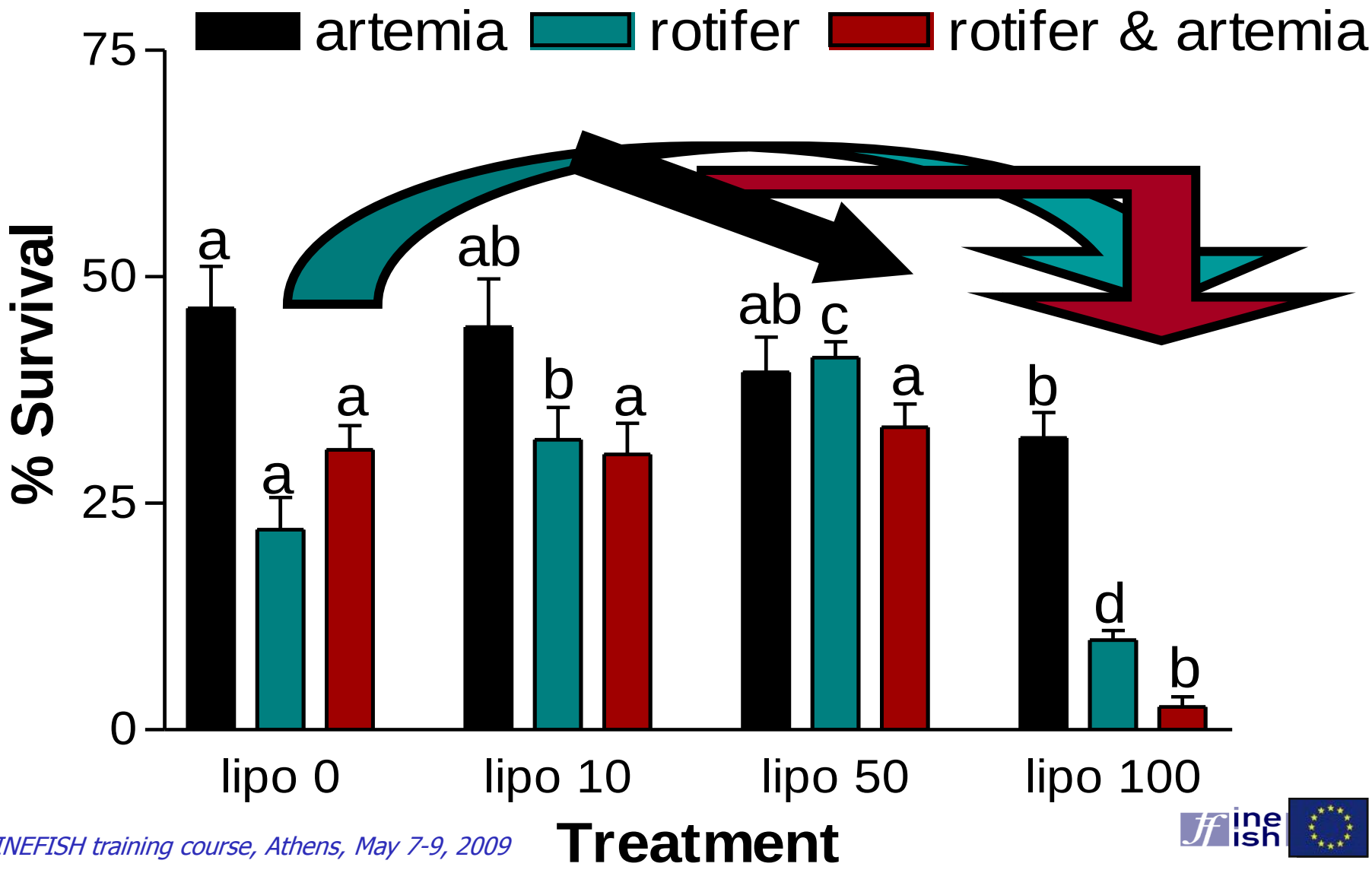
Yolk sac stage — **Rotifers** — **Artemia nauplii**

Experiment	Days 4-19	Days 20-34
1	Rotifers at various levels of Vit A enrichment	Un-enriched artemia nauplii
2	Un-enriched rotifers	Artemia nauplii at various levels of Vit A enrichment
3	Rotifers at various levels of Vit A enrichment	Artemia nauplii at various levels of Vit A enrichment

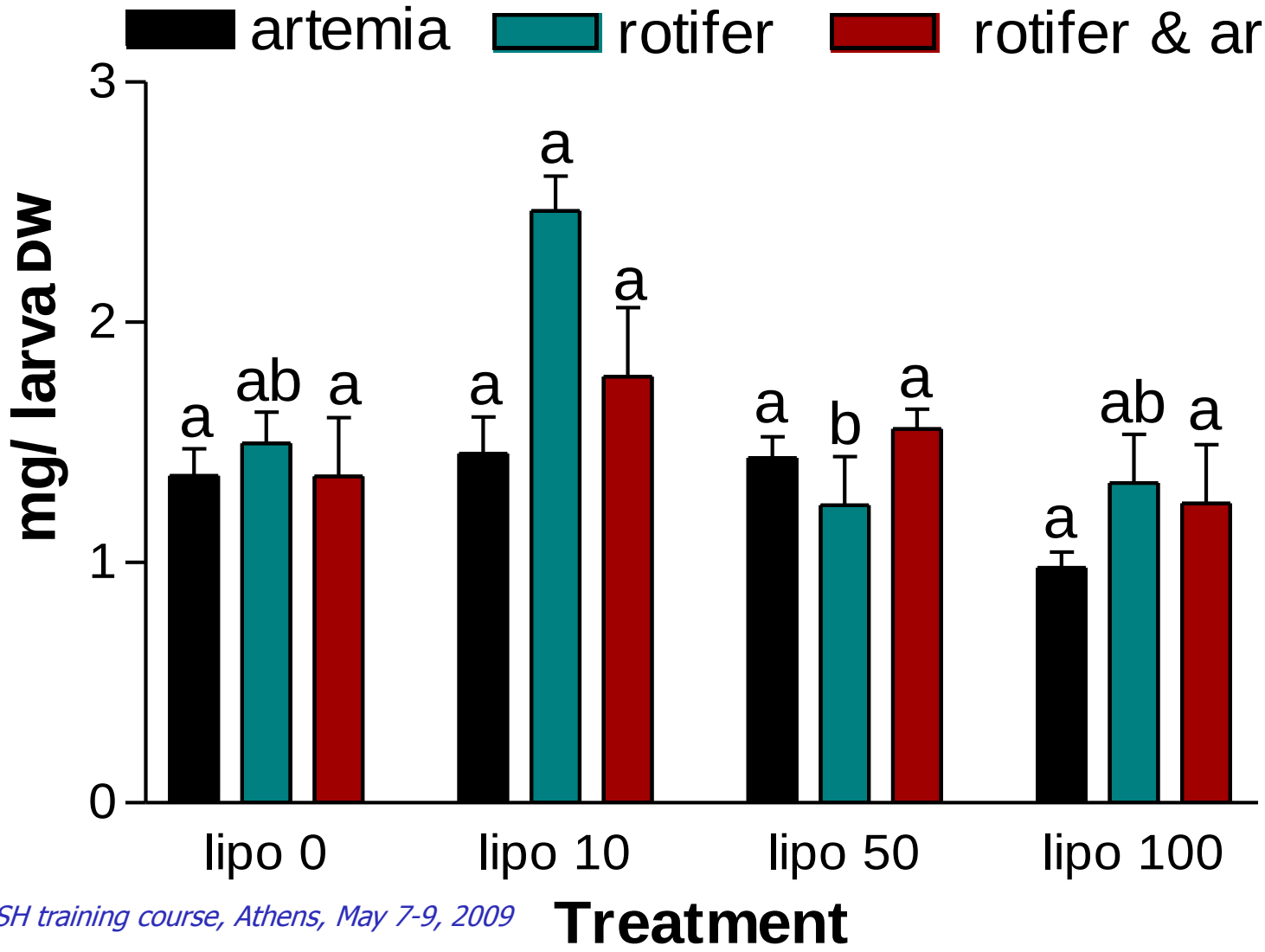
In order to observe late appearance of deformities, fish were maintained on a standard diet until the age of 120 d

Results

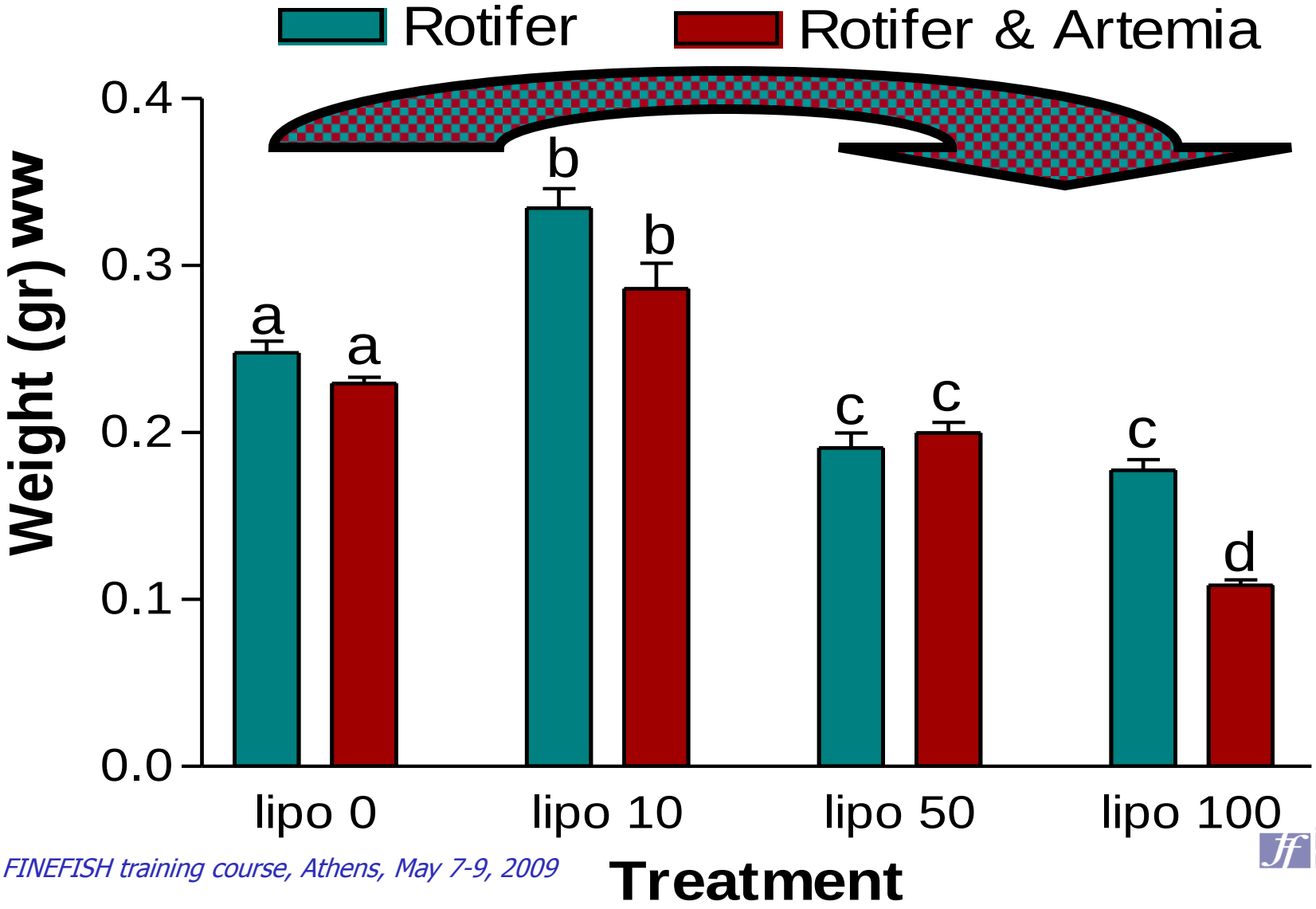
Survival rate of sea bream larva - 34 DPH



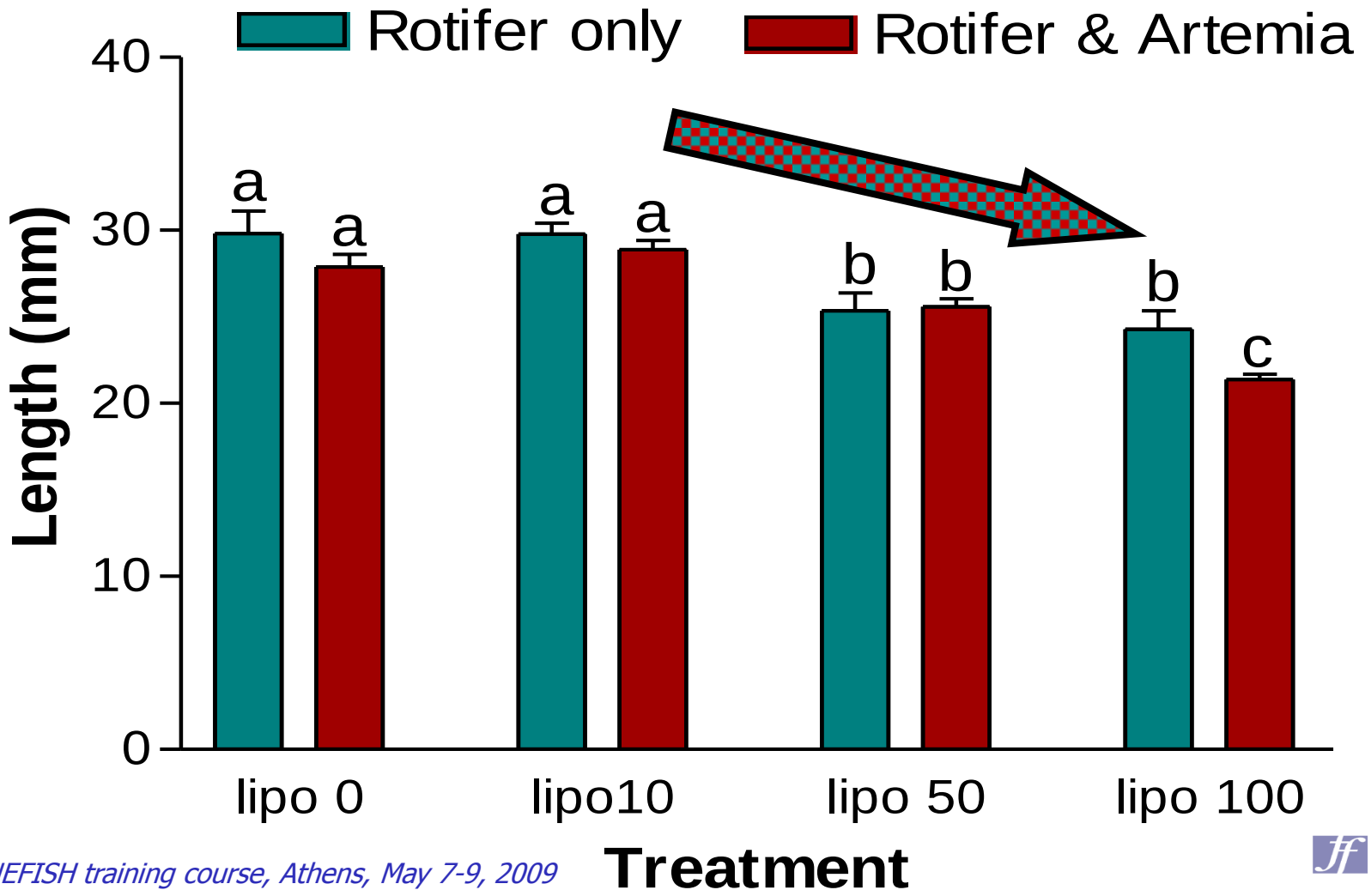
Mean weight of sea bream larva -34 DPH (vitamin A during rotifer & artemia stage)

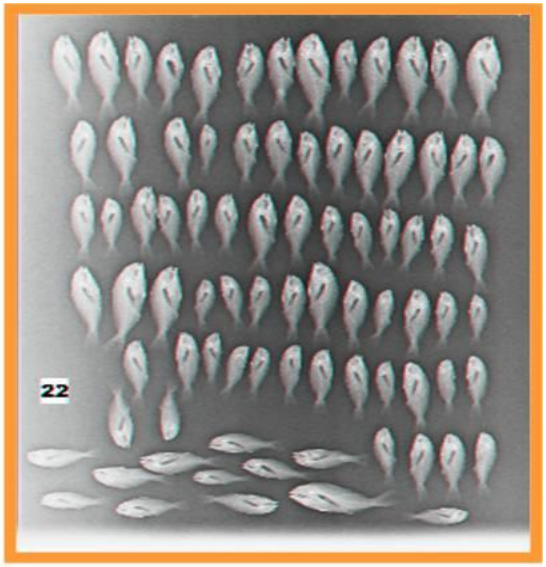


Mean weight of sea bream larva -70 DPH (vitamin A during rotifer & artemia stage)



Total length of sea bream larva - 70 DPH (vitamin A during rotifer & artemia stage)

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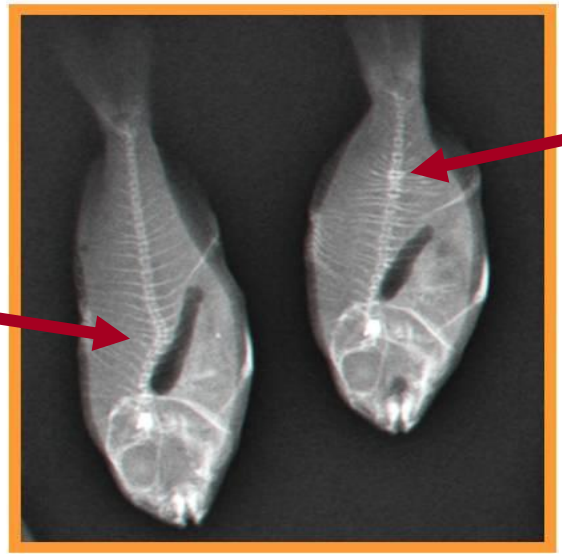
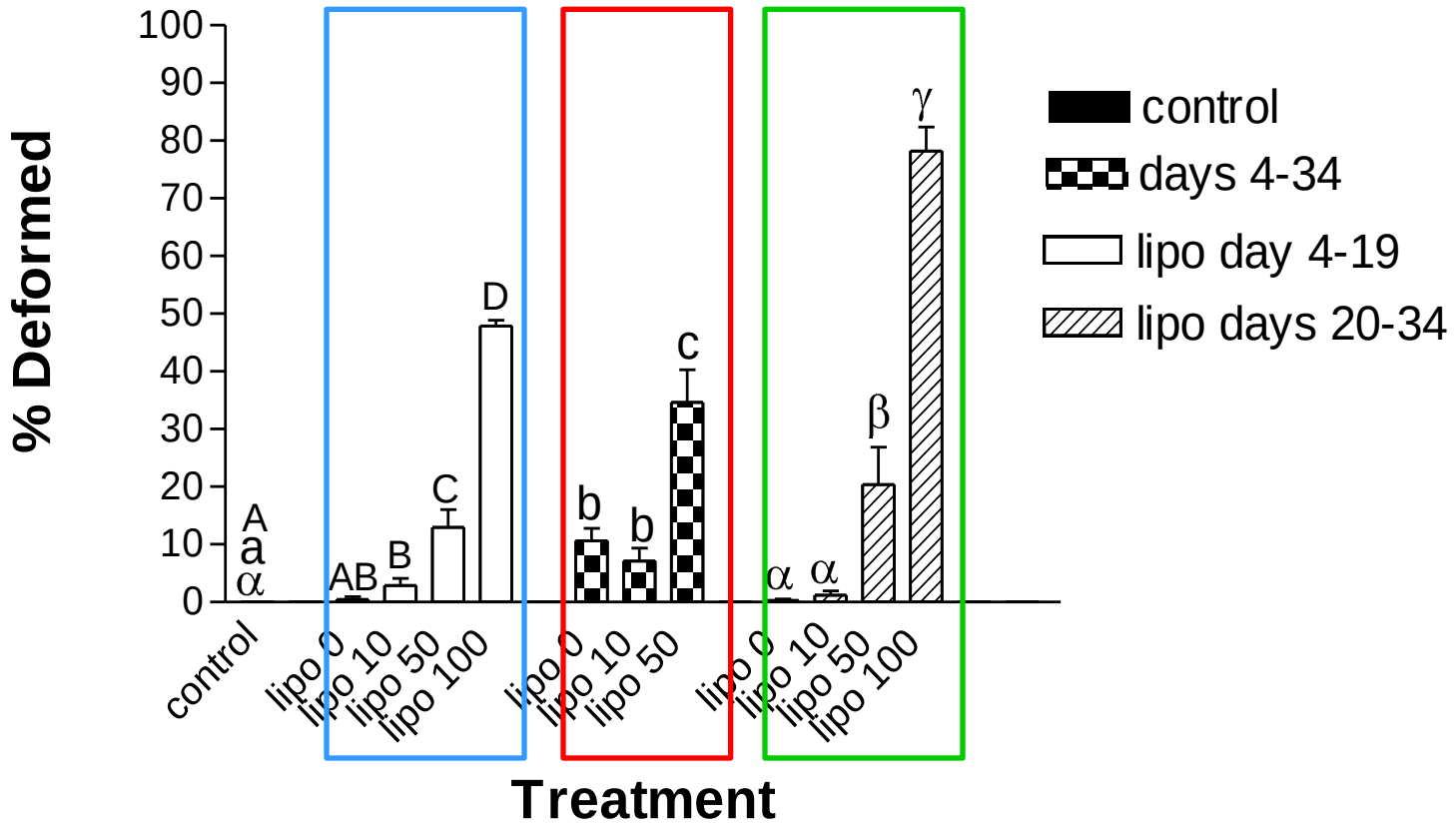
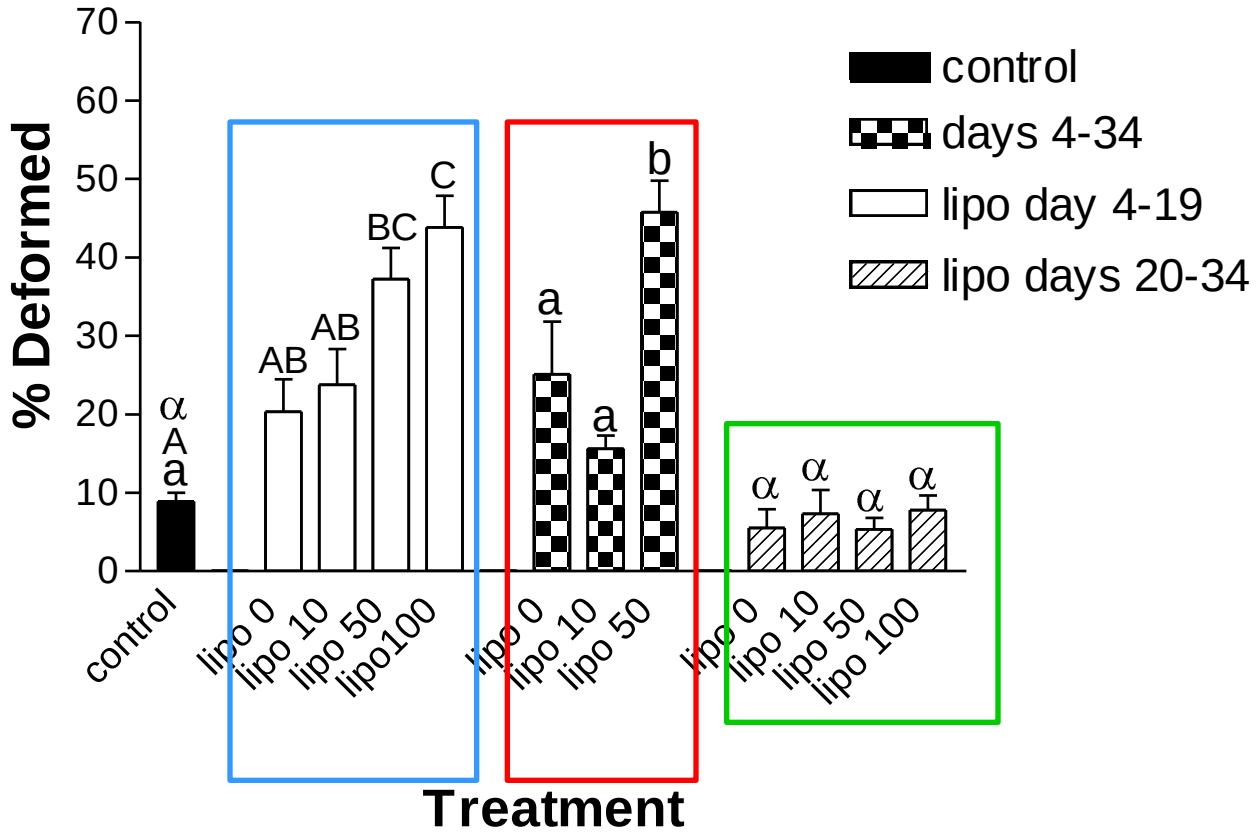


Skeletal (vertebral) deformations at 115 dph.



The effect of dietary supplementation of graded Vit A doses and different stages of the larval life (4-19 dph=rotifer stage; 20-34 dph= Artemia stage; 4-34 dph= rotifer and artemia stage)

Cranial deformations at 115 dph.



The effect of dietary supplementation of graded Vit A doses and different stages of the larval life (4-19 dph=rotifer stage; 20-34 dph= Artemia stage; 4-34 dph= rotifer and artemia stage)

Preliminary conclusions-2

(sea bream experiment)

- High dietary Vit A levels affected seabream larvae development mainly at the earlier age of up to 19 DPH
  - Mainly affected growth in this exp.
  - Induced the lack of swimbladder
  - Induced high % of cranial deformities
- High dosage of dietary Vit A at a later age dramatically induced
  - skeletal malformations

Conclusions and Recommendations for vit. A

- High dietary vitamin A levels strongly affect early larval development (before day 20); the effect is less marked after this stage.
- Vitamin A influences ossification and morphogenesis
- The optimal dose for the early developmental stage should be ~ 15 µg/g DW

Sea bass

VITAMIN D & VITAMIN C

Role of Vitamin D

- Maintains calcium and phosphate homeostasis
- Protects skeletal integrity
- Its becomes active after 2 hydroxylations
  - (1, 25 dihydroxycalciferol)
  - Considered as an hormon
  - Its action needs a receptor (VDR)

→ Does the dose used in the vitamin mix adequate?

Experimental design

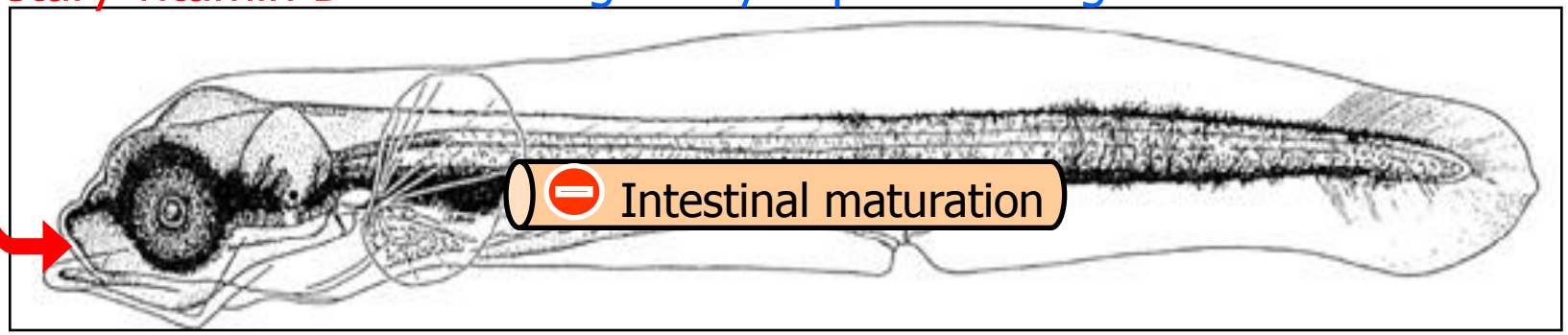
- 4 Vit Mix levels: 11.2 (VD-0), **27.60** (VD-1), 42 (VD-2) and 120 (VD-3) IU VD_3 per gram of diet
  - *Quite impossible to formulate a diet with vit D lower than 11.2 IU/g*
  - *VD-1 corresponds to ~8 times the NRC recommendation*
- 4 replicates per experimental group
- Duration: 45 days, and animals kept up to 2 g.
  - Growth and survival
  - Investigation of ossification process and deformities

Impact of Vit D

Low doses of dietary vitamin D

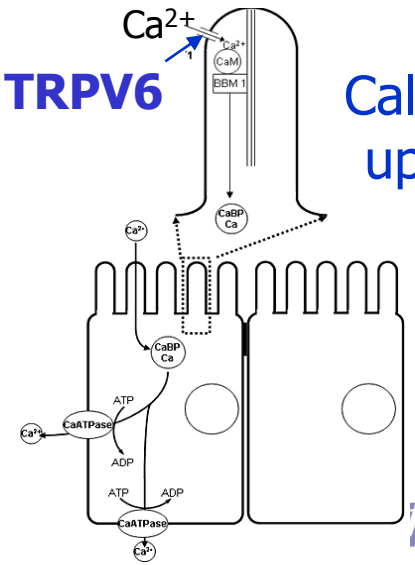
Enzymatic approach:

Negatively impacts on digestive tract maturation



qPCR approach:

low expression of TRPV6 gene



Calcium uptake

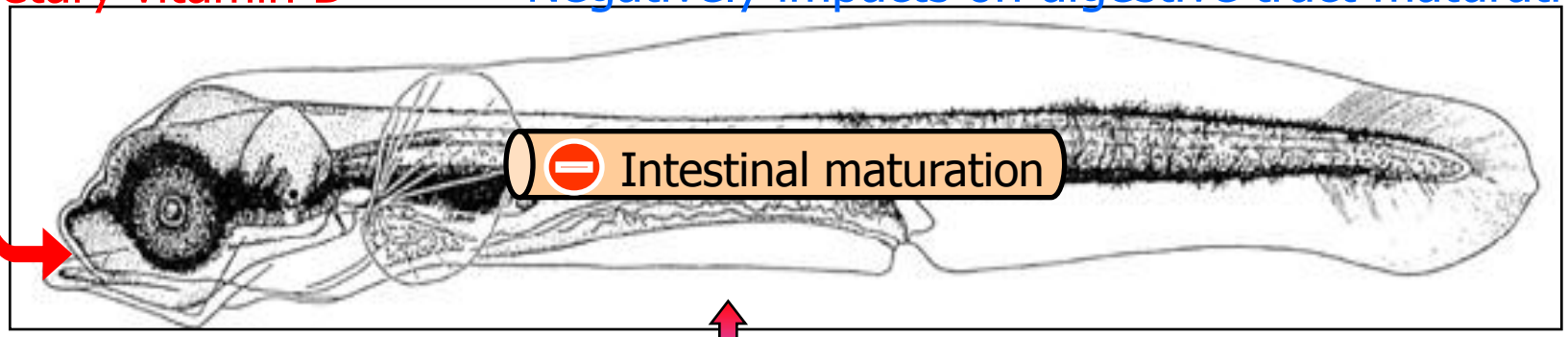
Impact of Vit D

Dietary vitamin D controls the ossification process through the regulation of intestinal maturation

Low doses of dietary vitamin D

Enzymatic approach:

Negatively impacts on digestive tract maturation



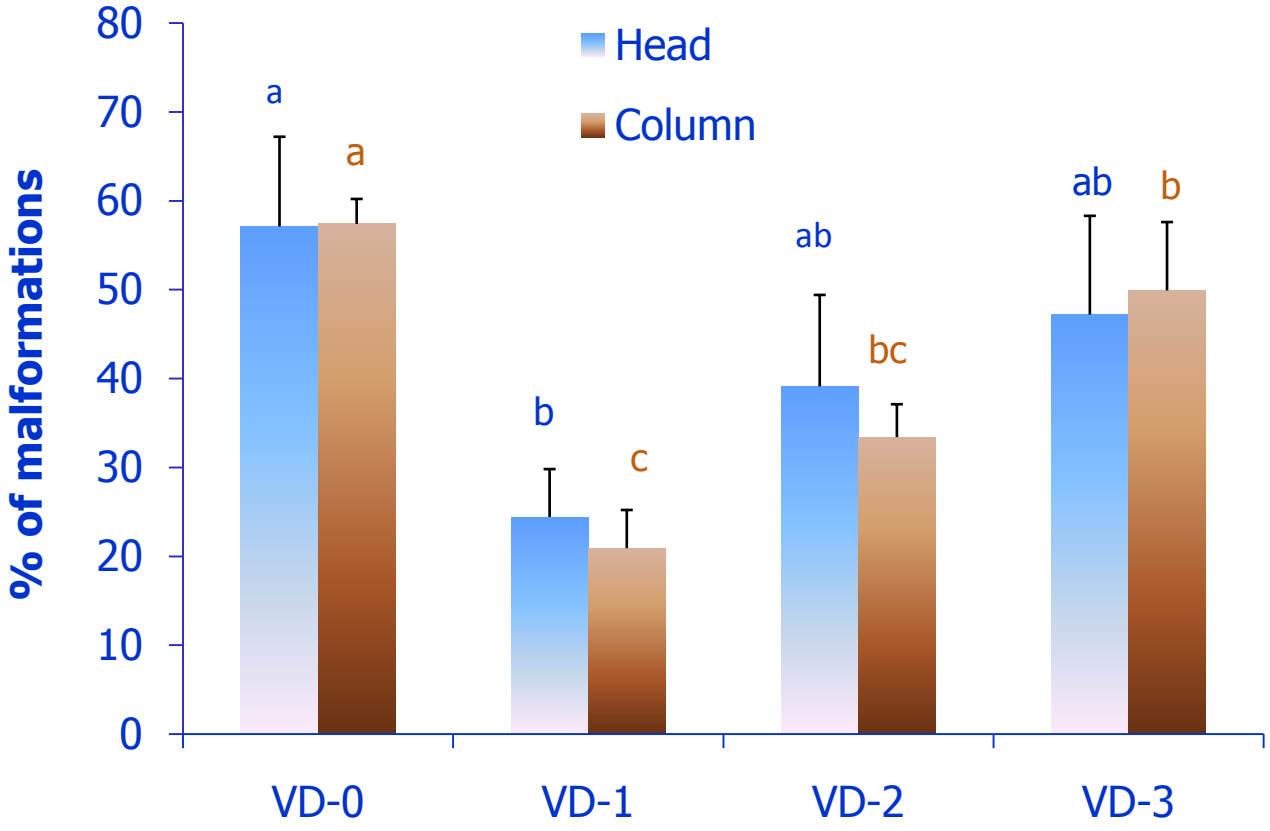
qPCR approach:

low expression of TRPV6 gene

Low calcium uptake

ossification/mineralization processes are delayed

Observed malformations



"Low" level of vit D negatively impacted on morphogenesis

VD-1 best compromise

Role of Vitamin C

- Vit. C: antioxydant, essential for collagen synthesis, and participates to several metabolic processes (immunity)

➡ Does the dose used in the vitamin mix adequate?

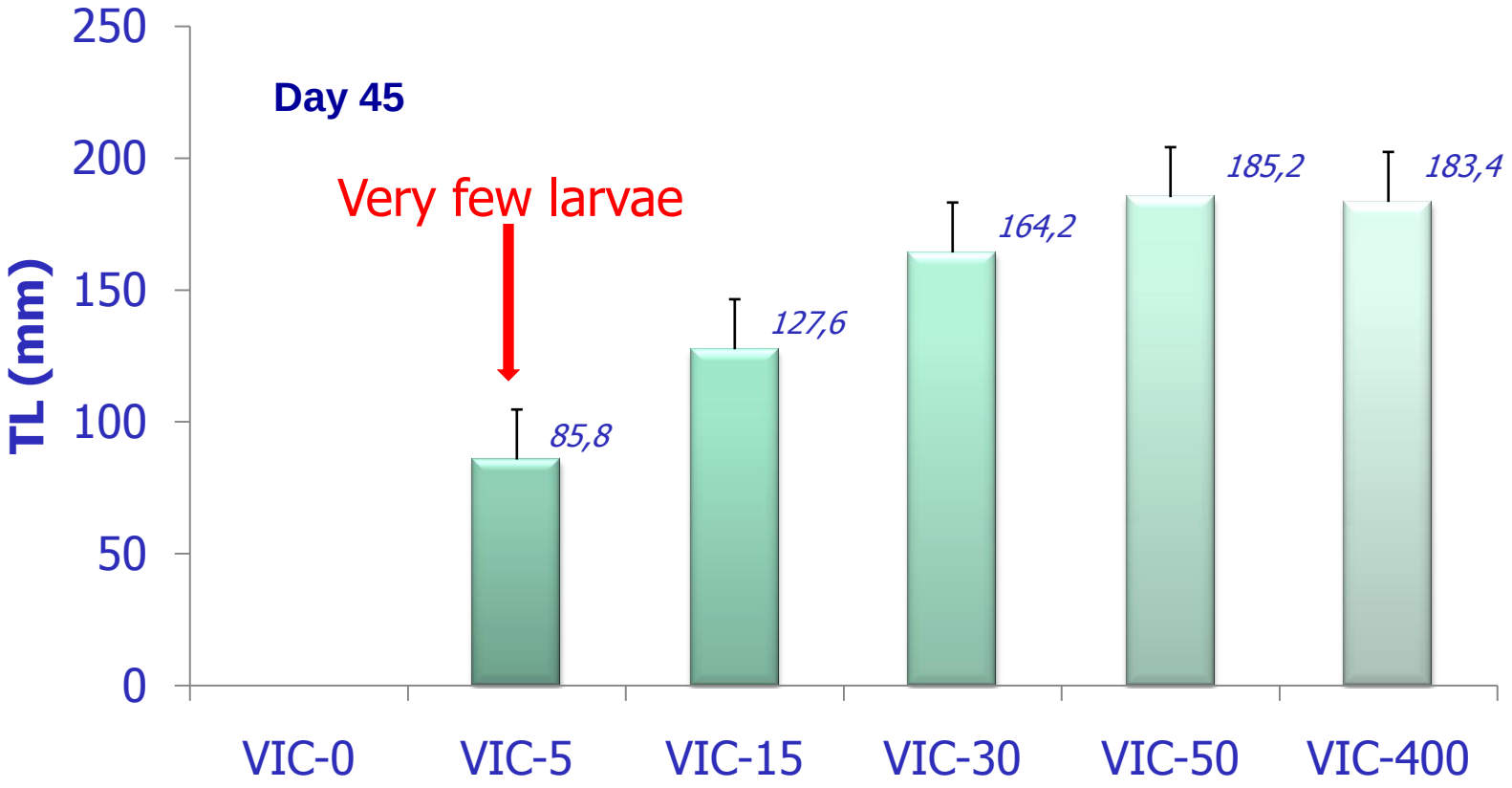
We decided to test the range from 0-400mg and to mostly fine tune 0-50mg.

Experimental protocol

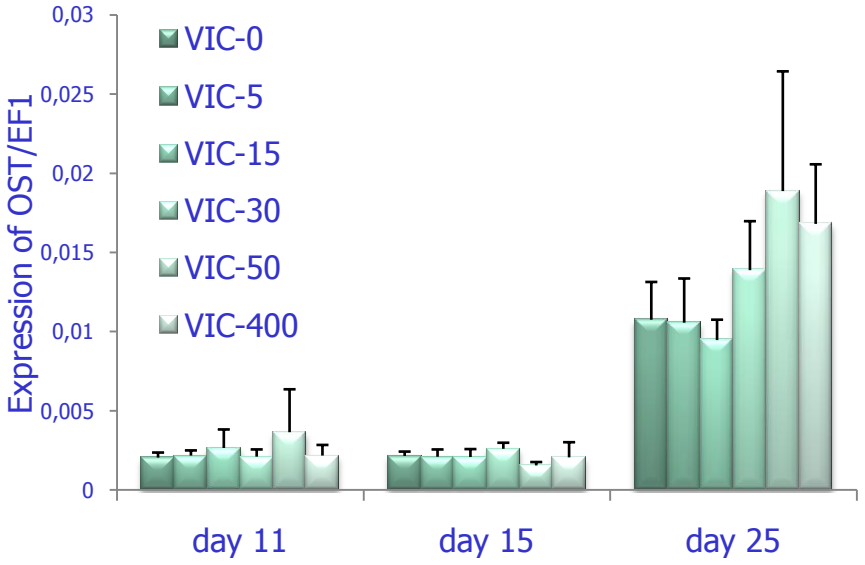
- 6 doses tested:
  - 0, 5, 15, 30, 50, 400 mg vit C/Kg aliment,
- 45 days
- 4 replicates
- Survival was not evaluated due to the elevated number of samplings
- Animals kept until 2 g.

Length of the larvae

VIC-0 and 5 did not survive after day 38; very poor survival for VIC-15 and VIC 30

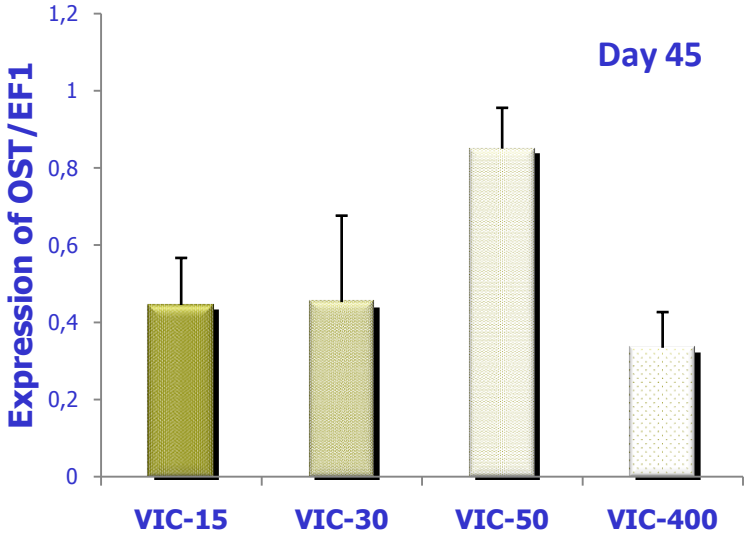


Osteoblast development

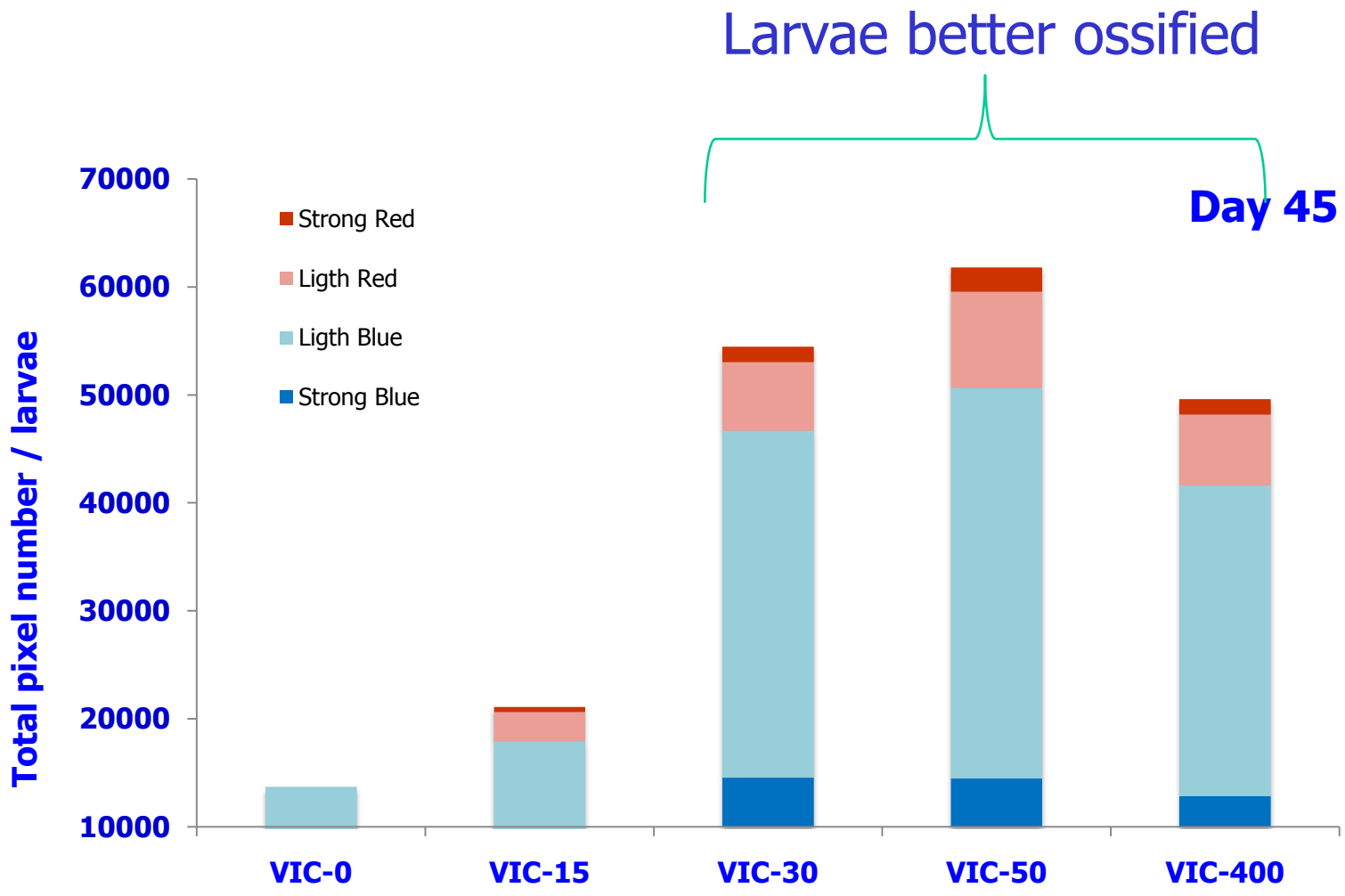


Osteocalcin was more expressed in VIC-50

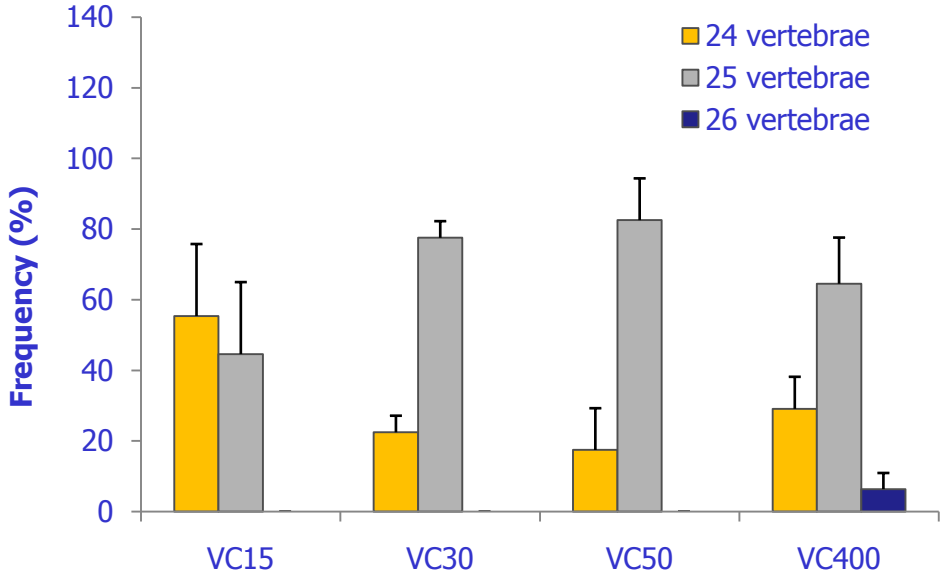
Osteocalcin better developped in VIC-50 and 400, and VIC-30 to a lesser extent



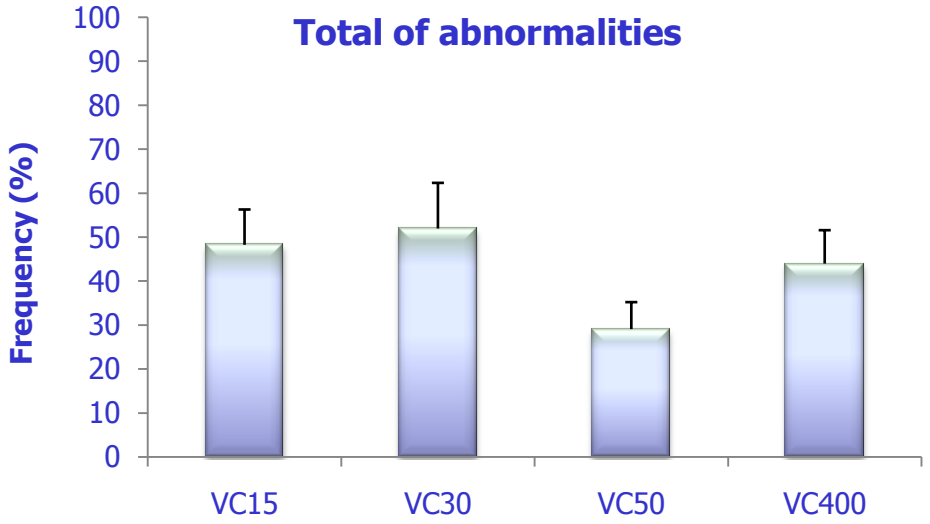
Ossification



Observed abnormalities



VIC-50 seemed to induced less malformations



Conclusions for Vit D and C

- Low vit D influences bone mineralization: risk limited for larval rearings considering the minimum doses present in the enrichment and feed incorporating fish meal.
- Low dietary vit C levels induced abnormalities
  - Due to a disturbed bone development and a less mineralization
  - The disturbance observed seemed to be linked to lipids (*we observed a stimulation of the markers of the adipocyte formation and of the Vit D pathway: results not shown*): consequence of a higher lipid oxydation?
- The higher Vit C dose also perturbed the bone formation and influence lipid metabolism: pro-oxydative effect?
  - Vitamin C requirement should be assessed concurrently to lipids?

Dietary vitamins as a causal factor for malformations in seabass and seabream

GENERAL CONCLUSIONS & RECOMMENDATIONS

Conclusions

- The dietary vitamin level clearly acts on morphogenesis
  - Sensible window of time during the 3 first weeks post hatching
  - Among the vitamins tested, Vit A has a potent teratogenic potential
  - Vit D has also an influence on bone mineralization but its disrupting capacity is limited considering the doses used in the larval feeding sequence.
  - Vit C indirectly acts on morphogenesis through its protective effect against lipid oxidation

Recommendations

- Check/Control the vitamin status of your feed…
  - Vitamin A should be lower than 100 ng/mg DW rotifer (~7 times less for inert feed), i.e. less than 450ng/ml enrichment of rotifers
  - Vitamin C should be around 50mg/g DW, but depends on lipid level
- Avoid dietary levels of EPA+DHA higher than 1.5 g/100g DW
  - ☞Could interfere with osteoblast development

Thank you!!