Ontogenic effects of early feeding of sea bass (*Dicentrarchus labrax*) larvae with a range of dietary n-3 HUFA levels on the functioning of PUFA desaturation pathways

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Abstract

Four replicated groups of sea bass larvae were fed XH (3.7% EPA+DHA), HH (1.7%), LH (0.7%) or XLH (0.5%) diets from d-6 to d-45 (XH*1*, HH*1*, LH*1*, XLH*1*; exp.1). After a subsequent one-month period feeding a commercial diet (2.7% EPA+DHA), the capacity of the four initial groups to adapt to an n-3 HUFA-Restricted diet (0.3% EPA+DHA; R-groups: XH*2*$_R$, HH*2*$_R$, LH*2*$_R$, XLH*2*$_R$) was tested for 35 days. Larval dietary treatments had no effect on larval and juvenile survival rates. Wet weight of d-45 larvae was higher in XH*1* and HH*1* ($P<0.001$), but the R-juvenile mass gains were similar in all treatments. Delta-6-desaturase (Δ6D) mRNA level was higher in LH*1* and XLH*1* at d-45 ($P<0.001$), and higher in LH*2*$_R$ and XLH*2*$_R$, with a significant increase at d-118. Concomitantly, PPAR α and β mRNA levels were higher in XLH*1* at d-45, and PPAR β and γ mRNA levels were higher in the XLH*2*$_R$ at d-118, suggesting possible involvement of PPARs in stimulation of Δ6D expression, when drastic dietary larval conditioning occurred. The low DHA content in phospholipid (PL) of LH*1* and XLH*1* revealed an n-3-HUFA deficiency in these groups. Larval conditioning did not affect DHA content in PL of R-juveniles. This study showed (i) a persistent Δ6D mRNA

enhancement in juveniles pre-conditioned with an n-3 HUFA deficient larval diet, over the one-month intermediate period, and (ii) brought new findings suggesting the involvement of PPARs in the Δ6D mRNA level stimulation. However such nutritional conditioning had no significant effect on juvenile growth and lipid composition.

Introduction

Worldwide supplies of fish oils and meals have reached their sustainable limits, forcing industries to look for alternative lipid sources for use in marine fish diets [(1)]. As terrestrial animal products are prohibited, there is great interest in aquaculture to produce fish better able to utilise vegetable feedstuffs. Vegetable products are rich in 18 carbon fatty acids (C_{18} FA) but do not contain C_{20-22} n-3 highly unsaturated fatty acids (n-3 HUFA), such as ecosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acids (DHA; 22:6n-3). These n-3 HUFA are required in the diet to provide the essential fatty acids for marine fish, as marine fish have a low capacity to produce C_{20-22} HUFA from C_{18} FA precursors, such as α-linolenic (18:3n-3) and linoleic (18:2n-6) acids [(2,3)]. Delta-6 desaturase (Δ6D) is the rate-limiting enzyme catalysing the first reaction of n-3 HUFA synthesis from 18:3n-3 and 18:2n-6 [(4)], but, as its activity is very low in marine fish [(3,5)], it could also limit the use of vegetable products by marine fish.

One solution could be to apply metabolic programming using nutritional conditioning during early larval stages, as already shown in mammals [(6)], in order to stimulate the FA desaturation pathways of n-3 HUFA synthesis in marine fish. We recently showed [(7,8)] that metabolism in sea bass (*Dicentrarchus labrax*) juveniles can be modulated by larval nutritional conditioning. The Δ6D mRNA level was enhanced in larvae fed a low n-3 HUFA diet (0.8% DM EPA+DHA), and this was retained in pre-conditioned juveniles fed an n-3 HUFA-restricted diet (0.5% DM EPA+DHA). Moreover, a slightly, but significantly, higher DHA content in phospholipid (PL) in pre-conditioned juveniles was measured. However, the larval conditioning did not significantly affect growth performance of juveniles in terms of weight and survival rates, suggesting that larval nutritional stimulus was not sufficient to induce further long-term effects.

The aim of the present study was to determine the range of dietary n-3 HUFA content that would elicit effects on desaturation/elongation pathways for n-3 HUFA synthesis and whether the effect could be amplified. Thus, a large range of n-3 HUFA dietary content (0.5-

3.7% EPA+DHA) was used during the larval stage, followed by a severe n-3 HUFA-restricted diet (0.3% EPA+DHA) during the juvenile period.

Materials and methods

Rearing conditions and experimental design

Larval conditioning: Experiment 1

Three days post-hatching sea bass (*Dicentrarchus labrax*) larvae were obtained from a commercial hatchery (Gravelines, France), and experiments were conducted at the Ifremer-Brest facility (Brest, France). Larvae were distributed in 20 conical fiberglass tanks (35 l; initial stocking density: 60 larvae l^{-1}, *i.e.* 2500 larvae $tank^{-1}$) and temperature was progressively increased from 13.5°C to 19°C within 2 days. All groups were fed microparticulate diets from mouth opening at day 6 (d-6) to d-45. Four experimental diets differing only in their n-3 HUFA content were tested: XH (3.7% EPA+DHA on a DM basis); HH (1.7%); LH (0.7%) and XLH (0.5%) (Table 1). The different n-3 HUFA contents were obtained by the incorporation of soybean oil in LH and XLH diets and by an inverse proportion gradient of soy lecithin and marine phospholipid. Four tanks were fed the HH or XH diets and six tanks were fed the LH or XLH diets. The four experimental conditions were XH*1*, HH*1*, LH*1* and XLH*1*. The rearing conditions were as described previously [7].

Juvenile period: Experiment 2

The larval period was followed by an intermediate period of one month (d-46 to d-77), during which the four groups were separately held at 19°C and fed a commercial diet with 2.7% EPA+DHA (DM basis), corresponding to the mean between the XH (3.7% EPA+DHA) and HH (1.7% EPA+DHA) diets. The four experimental groups were XH*2*, HH*2*, LH*2* and XLH*2*. The d-77 juveniles of each group were anaesthetised (ethylene-glycol-monophenylether, 0.15‰) and selected fish were randomly distributed in 60 l square tanks (180 fish per tank). The fish were acclimatised to the experimental unit for 6 days (d-77-83) at 19°C, and two experimental isolipidic and isoproteic diets differing in their n-3 HUFA content by the incorporation of either rapeseed oil or cod-liver oil (Table 2), were progressively introduced. Four replicate groups per initial condition were fed the experimental n-3 HUFA-Restricted diet (0.3% EPA+DHA); termed "R-groups" ($XH2_R$, $HH2_R$, $LH2_R$ and $XLH2_R$). Two other replicated groups per initial condition were fed an n-3 HUFA-rich diet

(1.4% EPA+DHA, *i.e.* 2-fold higher than the 0.7% EPA+DHA requirement defined for sea bass juveniles [9]) and used as control "C-groups" (XH*2*$_C$, HH*2*$_C$, LH*2*$_C$ and XLH*2*$_C$). The rearing conditions of juveniles were as described previously [8]. The experiment started when all groups were fed entirely on the HUFA-restricted or -rich diets (d-83, *i.e.* t0), and lasted until the final weights of all fish were increased at least two-fold (d-118; *i.e.* t35).

Sampling procedures

Experiment 1

For larval growth assessment and lipid composition, samplings were performed on 12 h fasted larvae at d-45, corresponding to the end of the larval period (when all enzymatic and molecular functions are established). For molecular analyses, intermediate samplings were also performed at d-10, d-17 and d-25.

Weight was monitored by sampling 30 larvae in four tanks per condition (120 larvae per condition; n=4). After a minimum preservation period of three weeks in 4% seawater formalin, larvae were individually weighed, pooled and dried for 24h at 105°C to estimate the dry weight of each group (n=4). Final biomass (mg.l^{-1}) was the larvae mean wet weight per survival rate at d-45 (n=6 for XLH*1* and LH*1* and n=4 for XH*1* and HH*1*). The survival rate was the ratio final/initial number of larvae in each tank, minus the number of larvae sampled (n=6 for XLH*1* and LH*1* and n=4 for XH*1* and HH*1*).

Measurement of mRNA level of genes involved in digestive functions and lipid metabolism (delta-6 desaturase Δ6D, and peroxisome-proliferator activated receptors alpha PPAR α, beta PPAR β and gamma PPARγ) was performed on 100 mg of larvae at d-10 and d-17 and on about 300 mg of larvae at d-25 and d-45, in four tanks per condition (n=4). Larvae were conserved in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) (1 ml for 100 mg of larvae) at –80°C pending analysis.

For lipid analysis, 50 pooled larvae from each of four tanks per condition (n=4) were weighed and conserved at –80°C pending analysis.

Experiment 2

Juveniles were anaesthetised before sampling. For all samplings, n=4 and n=2 for R-and C-groups, respectively. A HH*2*$_C$ tank was lost at d-90, inducing n=1 for this group at d-90, d-104 and d-118.

Growth was estimated by weighing individually 50 fish (± 0.1 g) at d-83 (t0), d-90 (t7), d-104 (t21) and d-118 (t35) in all tanks. The survival rate was estimated as for larvae. The daily growth index (DGI; %) between t0 (d-83) and t35 (d-118) was calculated as follow:

$$DGI_{\text{d-83-118}} = 100 \times (\sqrt[3]{w_{\text{d-83}}} - \sqrt[3]{w_{\text{d-118}}}) \text{ day}^{-1}$$

Measurement of mRNA level of genes involved in lipid metabolism (Δ6D, and PPAR α, β and γ) was performed on R-groups on 10 pooled fish from each of four tanks per condition (40 fish per condition) at d-83, d-90, d-107 and d-118. They were immediately frozen in liquid nitrogen and stored at –80°C until assayed.

Lipid analyses were performed on C- and R-groups. Six pooled fish per tank were taken at d-83 and 10 pooled fish per tank were taken at d-118. They were individually weighed, frozen in liquid nitrogen and stored at –80°C pending analysis.

Analytical methods

Gene expression

Expression of Δ6D, and PPAR α, PPAR β and PPAR γ genes was performed on whole body for larvae and on liver for juveniles. Dissections of frozen juveniles were conducted on a glass plate maintained at 0°C. The whole liver was isolated and the gall-bladder removed because bile can destroy RNAs. Gene expression measurements of each sample were performed on 200 mg of homogenised pooled livers (Polytron® PT 2100 Bioblock®). Total RNA was extracted from total larvae and livers using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNAs were obtained in duplicate from total RNA (1 µg) using Quantitect Reverse Transcription® kit with integrated removal of genomic DNA contamination (QIAGEN® GmbH, Hilden, Germany). Real-time PCR was performed using the iCycler iQTM® (Bio-Rad® Laboratories Inc., Hercules, CA, USA) as described in our previous studies [7,8]. The specificity of forward and reverse primers for each gene was checked by sequencing the amplicon (Eurogentec, Labège, France). Primers for Δ6D, PPAR α and PPAR β were as described previously [7]. Those for PPAR γ were 5'-3': CAGATCTGAGGGCTCTGTCC and 3'-5': CCTGGGTGGGTATCTGCTTA. Real-time PCR efficiencies were determined for each gene from the given slopes in Bio-Rad® software (iCycler iQ™ Real-Time Detection System Software, Bio-Rad® Laboratories Inc., Hercules, CA, USA), according to the equation 1:

$$E=10^{[-1/slope]}$$

To determine the relative quantity of target gene-specific transcripts present in the different samples, expression ratios (R) were calculated according to the following formula (2):

$$Ratio=[(E_{gene})^{\Delta CT\ target\ gene\ (mean\ control-mean\ sample)}]/[(E_{EF1})^{\Delta CT\ EF1\ (mean\ control-mean\ sample)}]$$

where “E” is the PCR efficiency and “mean sample” corresponds to triplicate average. The HH*1* and HH*2* samples were used as the standard group for larval and juvenile experiments, respectively, because they are close to the rearing condition in fish farming. Elongation factor 1 α (EF1 α) was used as the reference gene [10] as its expression is constant during activation and proliferation of cells [11].

Fatty acid composition

Whole frozen larvae were homogenised at 0°C using a Polytron® (PT 2100 Bioblock®, Illkirch, France), while whole frozen juvenile were homogenised rapidly with a Hobart® mixer (Sydney, Australia) in order to keep a low temperature and then, more accurately using a Polytron® (PT 2100 Bioblock®, Illkirch, France). Lipid analyses were performed on a representative portion (~1 g and ~5g for larvae and juvenile samples respectively) and ~3 g were taken for dry weight measurement (105°C; 24h). Assays were conducted on one larval sample, while for juveniles they were performed on duplicates at d-83 and at d-118.

Extraction of total lipid (TL), separation of neutral (NL) and polar lipid (PL), preparation of fatty acid methyl esters (FAME) and separation of FAME were performed on larvae and juveniles as described in our previous study [7,8]. Each chromatogram was visually controlled on the computer using an amplification of the baseline in order to check the peak shape and quality of integration by the computer program. Internal standard (tricosanoic acid 23:0) was used quantify FAME in TL and NL on a fish fresh matter basis, and was added to a weighed known quantity of larvae before the TL extraction, while it was added before the TL and PL FAME extraction for juvenile lipid analysis. The results of individual FA compositions were expressed as percent of total identified FAME.

Chemical analyses of feed were performed in duplicate for each sample according to AOAC [12] methods.

Statistical analysis

The data are presented as mean ± S.E. of the replicate groups. Before applying statistical tests using Statistica® (Tulsa, Oklaoma, USA), percentage data were transformed by arcsine

square root, and data for body weight, biomass and relative gene expression ratio were transformed by Ln. Effect of diet on growth performances, mRNA level for each sampling date and lipid composition was tested on means per tank using a one-way ANOVA, after control of equality of variances using Levene test. Effects of diet and age of fish on mRNA level were tested on means per tank using two-way ANOVA, after control of equality of variances using Chi-deux test. Effect of larval nutritional conditioning on mass gain of R-groups of juveniles, as well as between R-and C-groups was tested comparing curve slopes between t0 and t7; t7 and t21 and t21 and t35 by a one-way ANOVA. The Newman-Keuls multiple-range test was used to compare means in case of a significant effect ($P<0.05$).

Results

Experiment 1

Growth performances

Diet did not significantly influence larval survival rate (46.0±2.3 %), while the mean wet weight was more than 25% higher in XH*1* and HH*1* groups than in LH*1* and XLH*1* groups (Fig.1A; $P<0.001$). The mean final biomass of XLH*1* groups (892±108 $mg.l^{-1}$) was around 34% lower than that of XH*1* and HH*1* (1367±47 $mg.l^{-1}$; $P<0.01$), while biomass measured in LH*1* groups (1093±93 $mg.l^{-1}$) was not significantly different from the others (Fig.1B).

Gene expression

At d-10, the Δ6D mRNA level was similar in all groups (Fig.2A; 1.0±0.2). At d-17, LH*1* and XLH*1* groups exhibited higher values than XH*1* ($P<0.05$) but were similar to HH*1*. The difference between groups fed a low-HUFA diet (XLH*1*, LH*1*) and those fed a rich-HUFA diet (XH*1*, HH*1*) increased with time, and mean Δ6D mRNA level measured in XLH*1* and LH*1* groups was higher than that of XH*1* and HH*1* groups at d-45 ($P<0.01$).

One-way ANOVA analysis revealed that PPAR α, β and γ mRNA levels were not affected by diet from d-10 to d-25 (Fig.3A, 4A, 5A). At d-45, PPAR α and β mRNA levels were higher in XLH*1* groups than in others ($P<0.05$), while PPAR γ mRNA level was higher in XLH*1* groups than that measured in LH*1* larvae ($P<0.05$) but similar to that measured in XH*1* and HH*1* groups.

Lipid analysis

TL content in d-45 larvae was low (between 2 and 3% wet weight WW) and there were no significant differences between treatments (Table 3). Differences in NL composition of larvae at d-45 reflected those of the diets (Tables 1, 3). However, 18:3n-6 was significantly higher in NL of XLH*1* and LH*1* larvae than in NL of XH*1* and HH*1* larvae ($P<0.01$) independent of 18:3n-6 dietary content. HUFA: AA (arachidonic acid 20:4n-6), EPA and DHA contents were high in PL of d-45 larvae, and increased from XLH*1* to XH*1*, according to diet ($P<0.05$). The 18:2n-6 and 18:3n-3 contents were lower in PL of larvae than in diets, and decreased from XLH*1* to XH*1*, according to diet ($P<0.001$). Other PUFA were low and not directly related to diet composition: 18:3n-6 and 20:3n-6 were significantly higher in PL of LH*1* and XLH*1* than in PL of XH*1* and HH*1* ($P<0.001$ and $P<0.0.05$ respectively) and 20:2n-6 was lower in PL of XH*1* than in PL of other groups ($P<0.001$). The other intermediates in n-3 FA synthesis (18:4; 20:3, 20:4) were very low (0.12%; 0.06% and 0.20% of FAME, respectively) and their content was not different between groups (not presented in Table 3).

The PL content in d-45 larvae represented a major proportion of TL ranging from 41% to 47% with a significantly higher value in XH*1* fish than in LH*1* and XLH*1* groups ($P<0.05$). Accordingly, TL FA profiles of larvae (not presented here) showed intermediate percentages between those obtained in NL and PL.

Experiment 2

Growth performances

Juvenile survival rate (98.3±0.25%) was not affected by larval nutritional conditioning. There was no significant difference in weight increase from d-83 (t0) to d-118 (t35) in the four R-groups (2.2±0.02 g). The mass gain was similar between R-groups (Fig.6) and C-groups (not presented) from t0 to t7 (NS differences in curves slopes). From t7 to t21, mass increase was significantly higher in R-groups than in C-groups ($P<0.01$) and significantly lower from t21 to t35 ($P<0.01$). D-83 to d-118 daily growth index was not significantly different ($P=0.075$) between R- (1.13±0.02%) and C-groups (1.19±0.00%).

Gene expression

The mean Δ6D mRNA level was 2-fold higher at d-118 than any time-point earlier (Fig.2B; two-way ANOVA; $P<0.001$). One-way ANOVA performed at each sampling date indicated that Δ6D mRNA level was significantly higher in XLH2_R and LH2_R than in XH2_R

groups at d-83 ($P<0.05$), while XLH*2*$_R$ and HH*2*$_R$ showed similar values. At d-90, XLH*2*$_R$ and LH*2*$_R$ showed ~2-fold higher values than XH*2*$_R$ and HH*2*$_R$ ($P<0.05$) and at d-107, XLH*2*$_R$, LH*2*$_R$ and XH*2*$_R$ showed higher values than in HH*2*$_R$ ($P<0.05$). At d-118, the Δ6D mRNA level was lowest in XH*2*$_R$ groups (1.4±0.2) and highest in XLH*2*$_R$ and LH*2*$_R$ groups (4.7±0.5 and 5.6±0.6 respectively; $P<0.001$).

The mean PPAR α and β mRNA levels were globally higher at the end of exp.2 (d-118) than any previous time-point (Fig.3B and 4B; two-way ANOVA; $P<0.01$). Using one-way ANOVA, PPAR α and β mRNA levels were similar in all R-groups at d-83 and d-90. At d-107, PPAR α mRNA level was about 3-fold lower in XH*2*$_R$ than in other groups ($P<0.01$), while PPAR β mRNA levels were similar in all groups. At d-118, PPAR α mRNA level was similar in XH*2*$_R$ and LH*2*$_R$ groups, and about 50% lower than in HH*2*$_R$ and XLH*2*$_R$ groups ($P<0.05$). In comparison, PPAR β was significantly higher in XLH*2*$_R$ groups than in other groups ($P<0.01$). PPAR γ mRNA level was significantly lower at d-107 than at other sampling periods (Fig. 5B; two-way ANOVA; $P<0.001$). At d-83, it was more than 3-fold higher in LH*2*$_R$ groups than in others (3.4±0.6; $P<0.05$), while non-significant differences occurred between groups at d-90 and d-107. At d-118, XLH*2*$_R$ groups showed a significantly higher mRNA level than XH*2*$_R$ and HH*2*$_R$ ($P<0.01$).

Lipid analysis

The mean whole body TL content of R-groups was not significantly different between groups during the course of the experiment (Table 4). It was 4.4±0.4 % WW at d-83 and 9.2±0.2% at d-118. The PL content represented a higher proportion of TL at d-83 than at d-118 (29.5±1.1 *vs.* 13.4±0.9 % TL). At d-83 (*i.e.* after one-month feeding the commercial diet), the influence of diets observed during larval stage disappeared, FA composition was very similar in all groups, except for DHA, which was higher in NL of XH*2*$_R$ groups than in others (12.4±0.2 *vs.* 11.5±0.0 % FAME; $P<0.05$). At d-118, the DHA, EPA, AA, 18:3n-6 and saturated fatty acid contents in NL of R-groups were higher than in the R-diet ($P<0.05$), while 18:2n-6, 18:3n-3, MUFA and PUFA levels were lower (Tables 4, 2). DHA, EPA and 20:3n-3 were higher in NL of XH*2*$_R$ groups than in others at d-118 ($P<0.01$ and $P<0.05$ respectively). The 22:5n-3 content in NL of XH*2*$_R$ groups was higher than in LH*2*$_R$ and XLH*2*$_R$ ($P<0.05$). Other FAs in NL were not significantly different within R-groups. From d-83 to d-118, 18:2n-6, 18:3n-3 and MUFA content in NL increased by 45%, 75% and 41% respectively, while other FA, including DHA, EPA and AA decreased (11.9±0.2 *vs.* 2.4±0.1 % FAME for DHA). The 18:3n-6, AA, EPA and DHA contents were higher in PL of d-118-juveniles than in their

R-diet, while the contrary was observed for 18:2n-6 and 18:3n-3. From d-83 to d-118, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 18:3n-3 and MUFA content in PL increased, while others FA, including DHA, EPA and AA decreased. FA content in PL was not significantly different within R-groups at d-83, as well as at d-118, except for 20:4n-3, higher in $LH2_R$ groups than in others ($P<0.05$). The AA, EPA, DHA, saturated FA and PUFA were noticeably higher in PL than in NL at d-83 and d-118.

The fatty acid content of C-groups was related to C-diet (not detailed here). Their HUFA content was clearly higher than in R-groups (not detailed here). The AA, EPA and DHA contents in PL of C-groups were 1.9±0.0, 9.8±0.2 and 24.2±1.3 % FAME respectively. The low number of replicates did not allow a statistical evaluation within C-groups.

Discussion

The aim of this study was to elucidate whether the stimulation of desaturation/elongation pathways for n-3 HUFA synthesis in juveniles induced by a larval nutritional conditioning shown in a previous study [8] can be amplified using a large range of n-3 HUFA content (0.5-3.7% EPA+DHA) in the larval diet and a severe n-3 HUFA-restricted diet (0.3% EPA+DHA; R-diet) during the juvenile period.

As encountered in other studies [7, 13], diet composition had no significant effect on larval survival rates, while very low dietary n-3 HUFA content (XLH 0.5% EPA+DHA) led to decreased larval mass gain. Survival rates obtained were in agreement with a previous study [13], in which sea bass larvae reared at 19°C and fed a diet similar to HH*1* had a survival rate of 48% at d-38. The effect of high dietary HUFA content on mass gain could be the consequence of an elevated n-3-HUFA requirement for high cellular turn-over during the larval stage [14]. Larval mean weights obtained in this study were high at d-45, and the values obtained in HH*1* groups was much higher than found previously [13] in sea bass larvae reared in similar conditions. This could indicate the initial larvae were of high quality. As previously observed [8], larval conditioning did not affect growth performance of sea bass juveniles fed the R-diet, despite large differences in juvenile initial weight at the onset of the experiment 2, as a result of the different n-3 HUFA contents of the larval diets. The weight increase of R-juveniles was good in all groups, as it more than doubled in 35 days, and was not significantly different from that observed in C-groups during the first week of the experiment. However, the growth of R-groups seemed to be limited during the last period of the experiment. This

was in accordance with a significant growth retardation of sea bass juveniles fed different HUFA dietary contents for 7 weeks [9].

As observed earlier [7,8], the level of Δ6D mRNA was significantly higher in larvae fed a restricted n-3 HUFA diet during the larval stage (0.5 or 0.7% EPA+DHA), and in juveniles fed a low HUFA diet (0.3% EPA+DHA), following a transient feeding on a HUFA diet. This revealed that (i) Δ6D transcription could be modulated by the n-3 HUFA content of the diet, as observed in seabream [15] and, that (ii) conditioned juveniles were better than unconditioned fish in better developing desaturation processes in order to adapt to a low dietary HUFA content. The mRNA expression data were supported by the significant increase in 18:3n-6 measured in PL, as it is the Δ6D desaturation product of 18:2n-6, and could not have been obtained through the diet. These findings indicate that the increase in the level of Δ6D mRNA likely led to an increase in Δ6D enzymatic activity required for the first step of the bioconversion of 18 carbon FA to HUFA (20-22 carbons), and for the conversion of EPA to DHA [16]. Contrary to our previous study [8], the present results showed a persistence of Δ6D mRNA level in juveniles 30 days after feeding of the larval diets ceased, and beyond the intermediate period on a high HUFA diet. It may be a consequence of (i) the more restricted HUFA contents in the larval (0.5 and 0.7% EPA+DHA *vs.* 0.8%) and juvenile (0.3% *vs.* 0.5%) diets used, (ii) from the younger fish used (d-83 *vs.* d-151 at the beginning of experiment 2), or (iii) from a shorter acclimation period (30 days *vs.* 90 days).

Our results demonstrated that PPAR α and PPAR β genes, which are involved in FA catabolism and keratinocyte differentiation, showed (i) a higher mRNA level in d-45 larvae fed the lowest dietary n-3 HUFA content (0.5% EPA+DHA) and, (ii) this was maintained in d-118 juveniles in the case of PPAR β. These results were in concordance with the higher Δ6D mRNA level measured in these groups at the same times. According to several studies conducted in mammals [17], PPARs are involved, along with sterol regulatory element binding protein-1 (SREBP-1a and SREBP-1c), in the control of the Δ6D gene transcription. SREBP-1 binds to sterol regulatory elements (SREs), and mediates the suppression of the Δ6D gene by HUFA. In the present study, the concomitant increase of PPARs and Δ6D mRNA levels suggested that PPARs could be partly involved in modulating Δ6D gene expression in larval and juvenile sea bass. PPAR γ is involved in adipocyte differentiation and induction of lipogenic enzymes and, although its mRNA level was not significantly higher in larvae fed an n-3 HUFA-deprived diet, it was significantly higher in d-118 juveniles pre-conditioned with the lowest n-3 HUFA diet during the larval stage. This suggested that PPAR γ could also have a role in the stimulation of the Δ6D gene expression observed at the same time. The

stimulation of PPAR mRNA level was not significantly higher in juveniles pre-conditioned with the LH diet (0.7% HUFA dietary content), in spite of the significantly higher Δ6D mRNA level measured in these groups. We could hypothesise that the Δ6D gene could possibly be stimulated by PPARs when drastic nutritional conditions occurred, and that above a threshold, other mechanisms like those observed in mammals may be implicated, such as SREBP-1. This hypothesis is in concordance with previous results [8], which did not reveal any significant stimulation of PPARs, using a conditioning larval diet containing 0.8% EPA+DHA, while a higher Δ6D mRNA level was observed.

As the increase in PPAR and Δ6D mRNA levels observed in larvae fed a low HUFA diet were retained in juveniles fed a low HUFA diet, this indicated that (i) pre-conditioned fish were able to develop adaptation to low dietary HUFA content during juvenile period and that (ii) this adaptation could be the consequence of nutritional programming occurring during larval stage. Several existing biological mechanisms described in mammals could explain the "memory" of metabolic effects of early nutritional environments in juveniles [18]: Induced variations in organ structure, alterations in cell number, clonal selection, metabolic differentiation, hepatocyte polyploidisation and epigenetic modifications. In this study, memory of metabolic process in juveniles could be due to epigenetic modifications of the Δ6D and PPARs genes. Epigenetic modifications are modifications of DNA and covalent modifications of histones, which condition the accessibility of chromatin to transcription factors, facilitating the recognition of genes to be expressed or silenced, transiently or permanently, by these factors [18]. The hepatocyte nuclear factor 1 α (HNF1 α) has been identified as an homeoprotein expressed in liver, kidney, pancreas and digestive tract that could activate transcription through participation in the recruitment of the general transcription machinery to the promoter, or through the remodelling of chromatin structure and demethylation that would allow transcription factors to interact with their cognate *cis*-acting elements [19].

The relatively high level of n-3 HUFA measured in PL *vs.* NL of larvae and juveniles was in agreement with the preferential incorporation of these FA in PL contributing to the maintenance of PL quality [20]. Although AA, EPA and DHA were selectively incorporated in PL of larvae, low values were observed in PL of fish fed low n-3 HUFA diet (LH*1* and XLH*1*), revealing an n-3 deficiency in these groups. Even though growth was similar in LH*1* and XLH*1* larvae, HUFA content in PL was different within these groups, in accordance with values previously observed in d-45 sea bass larvae fed a diet with similar EPA+DHA content [7]. The XH*1* groups showed an exceptionally high DHA content in both PL and NL in d-45

larvae, which has rarely been observed in aquaculture, except in larvae fed on natural plankton or on rotifers enriched with DHA [21, 22]. The AA deficiency observed in larvae fed the LH*1* and XLH*1* diet, while its precursor 18:2n-6 increased in these groups, can be explained by low activity of Δ5D in these groups. This hypothesis was in accordance with the very low enzymatic activity of Δ5D compared to that of Δ6D measured in seabream *Sparus aurata* [23]. That 18:4n-3 was not increased could be due to the higher concentration of this fatty acid in larvae combined with the low concentration of its precursor (18:3n-3) in the diets used, as shown in microsomes of dogs and rats [24]. Moreover, as the level of 18:2n-6 is ten-fold higher than 18:3n-3 in the diets, its bioconversion could be stimulated in larvae even although Δ6D usually shows higher affinity with n-3 fatty acids than with n-6 fatty acids [25]. This suggested that production of 18:4n-3 could exist in n-3 HUFA-deprived larvae, even if it is not observable with the techniques used. Thus, the high level of n-6 fatty acids in the diet may mask effects on the n-3 HUFA synthesis pathway.

D-83 R-juveniles showed a similar composition in NL, while at d-118, several n-3 HUFA, including EPA and DHA, were present at a higher level in NL of XH*2*$_R$ juveniles than in others. This indicated that differences observed at d-118 could be the consequence of the growth dependent-dilution effect of initial (d-83) FA stores in the smaller fish [26]. The DHA content in PL of R-juveniles at d-118 (about 17%; $P<0.001$) was intermediate between the DHA content of n-3 deprived LH*1* and sufficient HH*1* larvae, and significantly lower than in C-groups (about 24%), and d-83 juveniles (about 27%). This was in agreement with a previous study [9], which showed that sea bass juveniles fed at or above requirement had a minimal DHA content in PL of around 20% of total FA. The FA content in PL of d-118 juveniles remained similar in all groups, except for 20:4n-3, which was at a higher level in XH*2*$_R$ groups than in others. In a previous experiment [8], a slightly higher DHA content in PL was found in juveniles conditioned with a n-3 HUFA deprived diet during the larval stage than in others, suggesting an enhanced capacity to adapt to a restricted-HUFA diet. A similar result was not obtained in the present study, which could be the consequence of technical differences between the two experiments, or to biological mechanisms. Irrespective, the two studies showed that the observed stimulation of Δ6D mRNA was not linked to an increase in PL n-3 HUFA content, and this could be due to the very low rate of desaturation already described for European sea bass, even when up-regulated by diet [27].

Conclusion

This study demonstrated an amplified stimulation of Δ6D mRNA induced by dietary n-3 HUFA deficiency in juveniles pre-conditioned with a low dietary n-3 HUFA content during the larval stage, and persisting in young juveniles. However, this did not have a noticeable influence on FA composition and growth performances in juveniles challenged with a HUFA restricted diet. Our results also suggested the involvement of PPARs in the regulation of Δ6D gene expression. Further studies concerning enzymatic activities of Δ6D and PPARs gene regulation are required to further investigate and understand the metabolic pathways for HUFA synthesis in marine fish.

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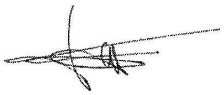
All the contributing authors know they are responsible for recognizing and disclosing any conflict of interest that could be perceived to bias their work, acknowledging all financial support and any other personal connections. There is no conflict of interest that authors should disclose, having read the above statement.

M. Vagner



J.H. Robin

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J. Person-Le Ruyet

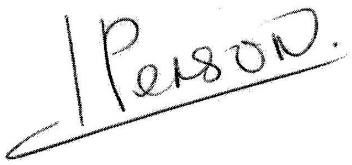

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Fig.1 ; Vagner et al.

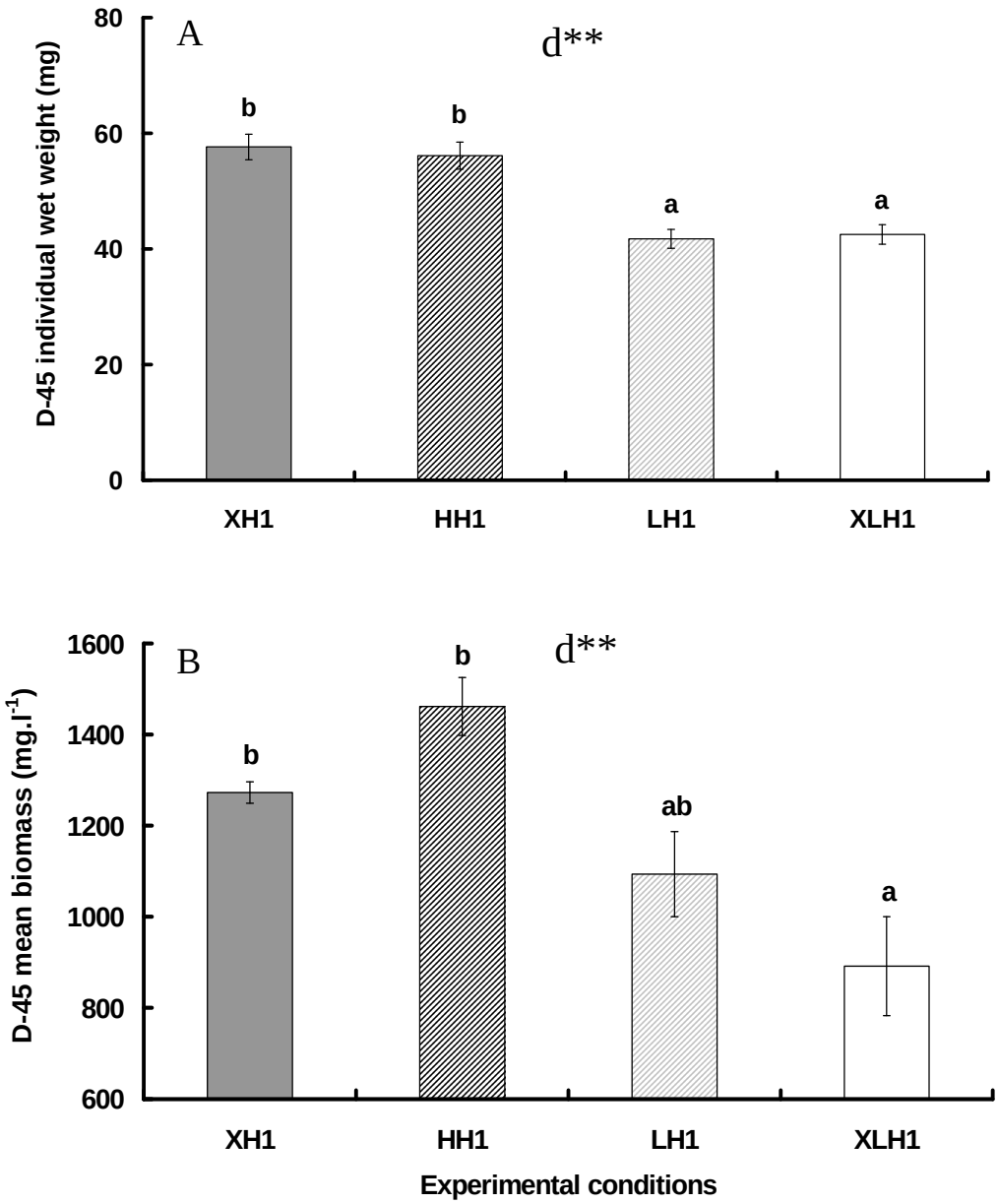

Fig.1. (A) D-45 mean larval wet weight (n=4) and (B) biomass (n=4 for XH1 and HH1 groups and n=6 for XLH1 and LH1 groups) at each experimental condition. Values are means ± SE and statistical significance of diet (d) is indicated (** P<0.01). Values not sharing a common letter are significantly different.

Fig.2 ; Vagner et al.

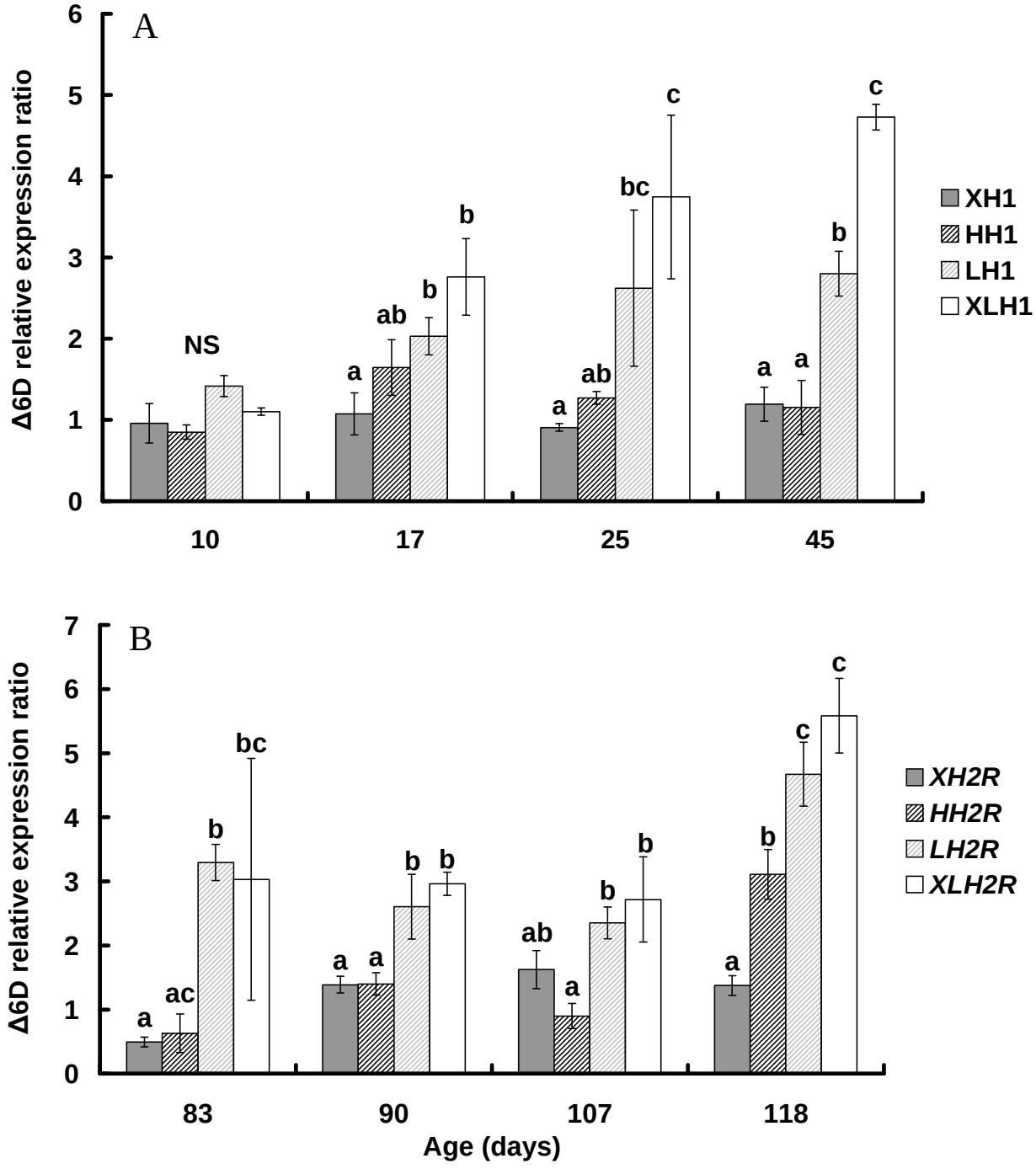

Fig.2. Mean Δ6 desaturase gene expression ratio (± SE) relative to HH*1* (A) and $HH2_R$ groups (B) according to time in larval (A) and juvenile (B) experiment and for each experimental condition (n=4). [a, b, c] differing letters denote significant difference for each date ($P<0.05$).

Fig.3 ; Vagner et al.

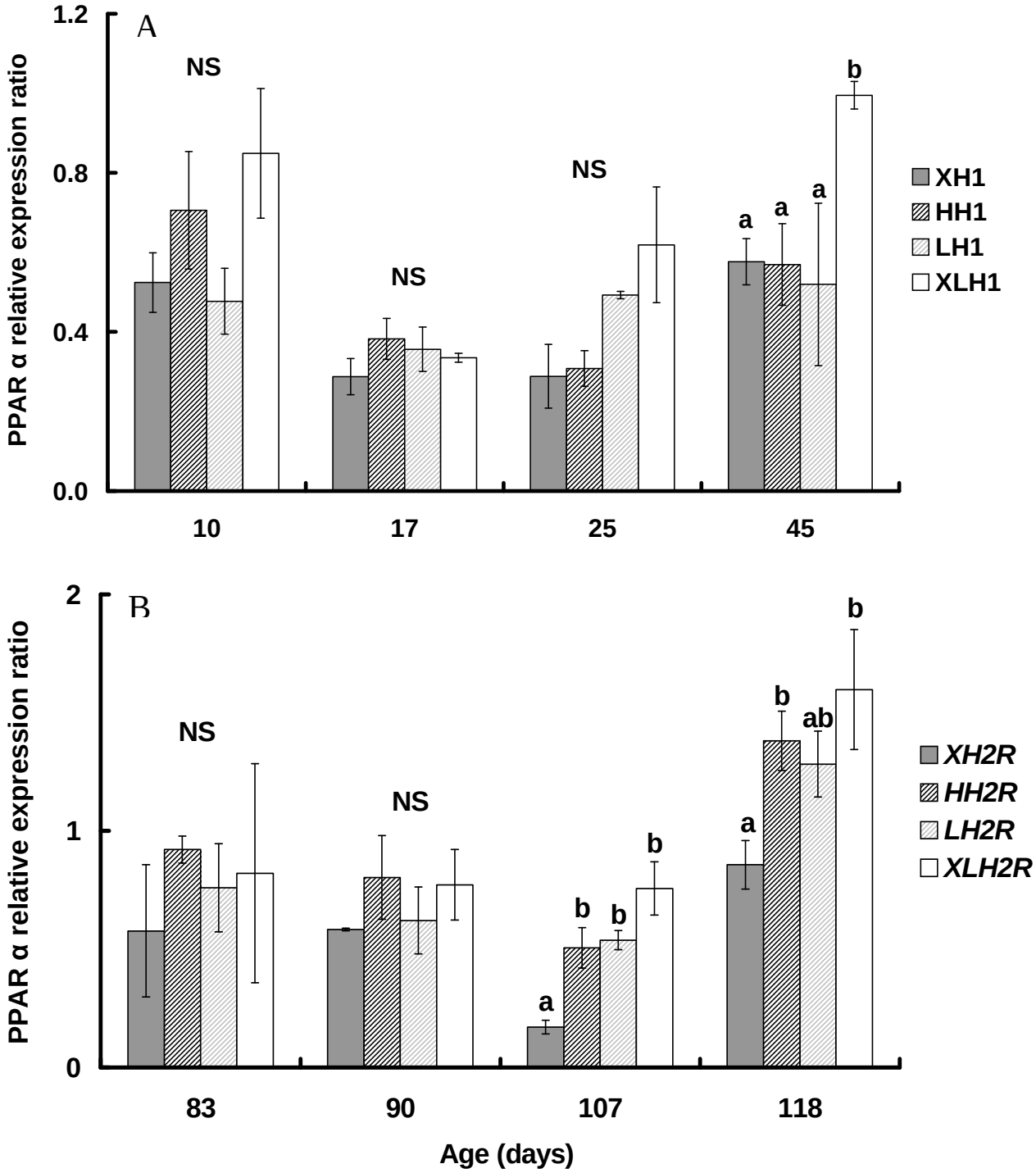

Fig.3. Mean PPAR α expression ratio (± SE) relative to HH*1* (A) and $HH2_R$ groups (B) according to time in larval (A) and juvenile (B) experiment and for each experimental condition (n=4). For each date, NS indicates non significant differences between groups and [a, b, c] differing letters denote significant difference at $P<0.05$.

Fig.4 ; Vagner et al.

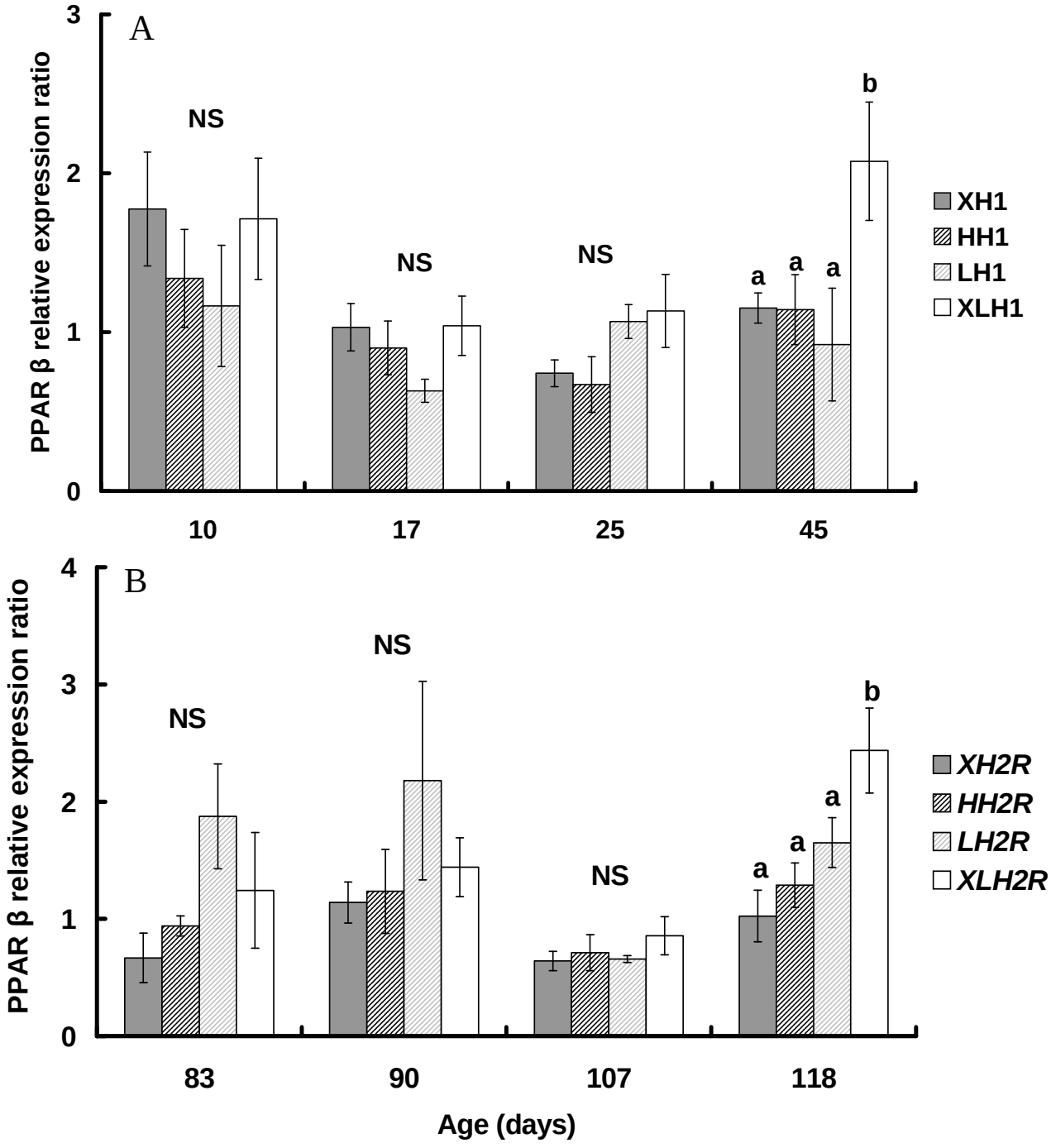

Fig.4. Mean PPAR β expression ratio (± SE) relative to HH*1* (A) and HH*2*$_R$ (B) according to time in larval (A) and juvenile (B) experiment and for each experimental condition (n=4). For each date, NS indicates non significant differences between groups and $^{a, b, c}$ differing letters denote significant difference at $P<0.05$.

Fig.5 ; Vagner et al.

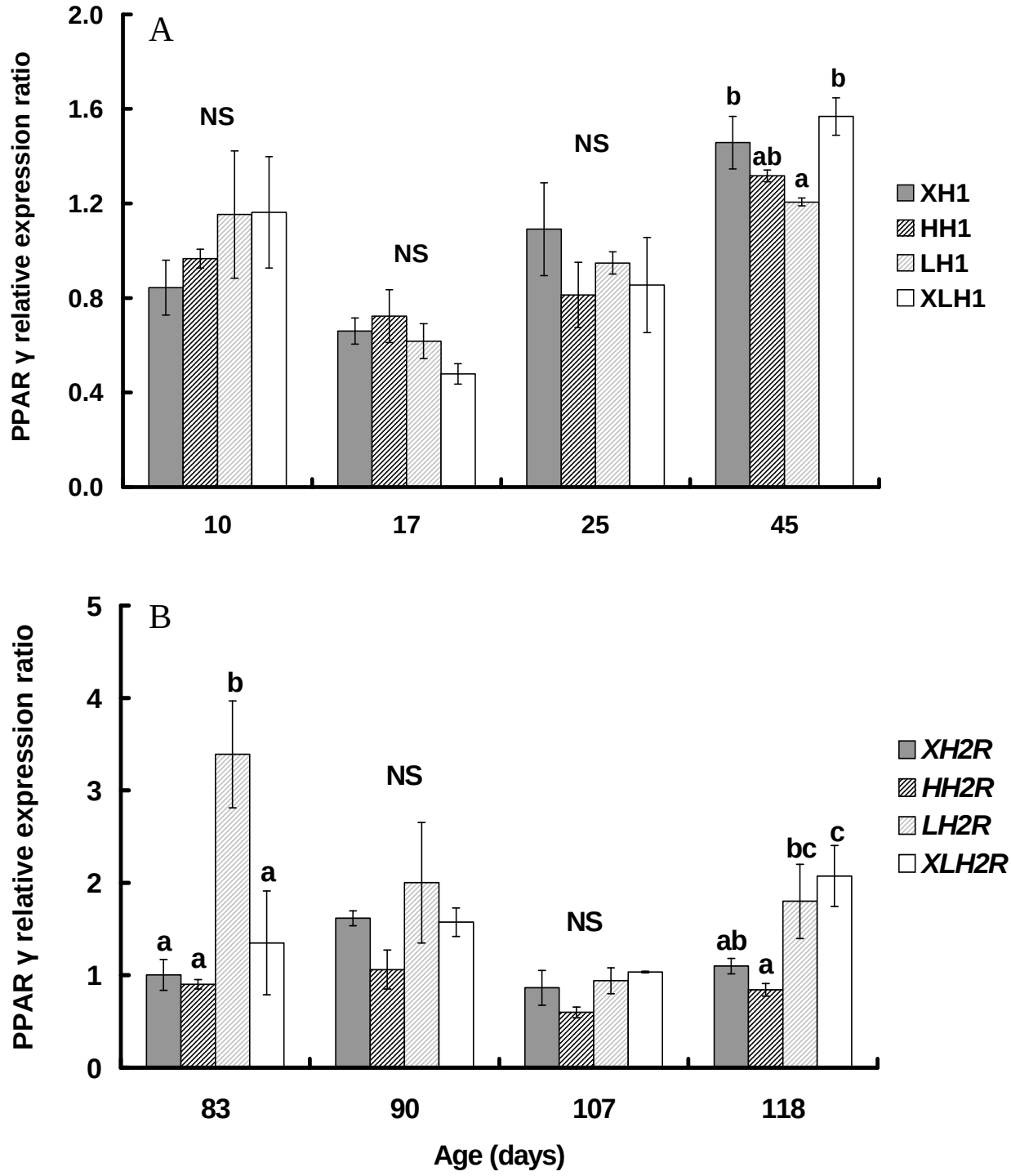

Fig.5. Mean PPAR γ expression ratio (± SE) relative to HH*1* (A) and $HH2_R$ (B) according to time in larval (A) and juvenile (B) experiment and for each experimental condition (n=4). For each date, NS indicates non significant differences between groups and $^{a, b, c}$ differing letters denote significant difference at $P<0.05$.

Fig.6 ; Vagner et al.

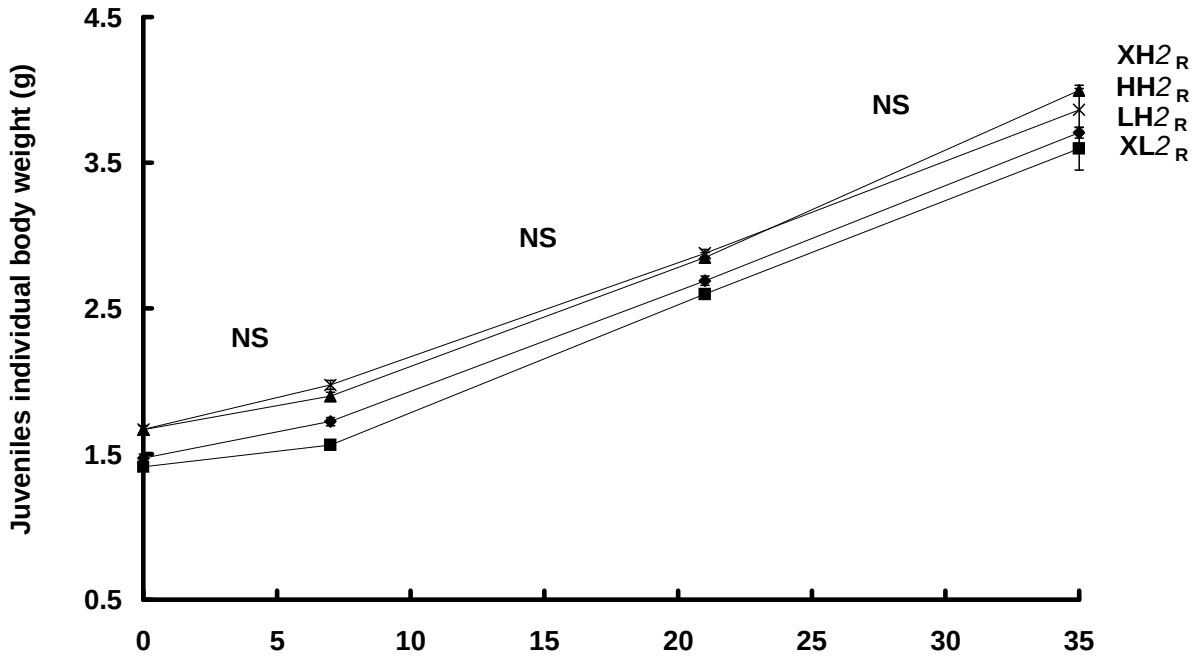

Fig.6. Mean fresh body weight (±SE) increase over time (d-83-118) for the 4 R-groups (n=4). NS indicates non significant differences between groups.

Fig.7; Vagner et al.

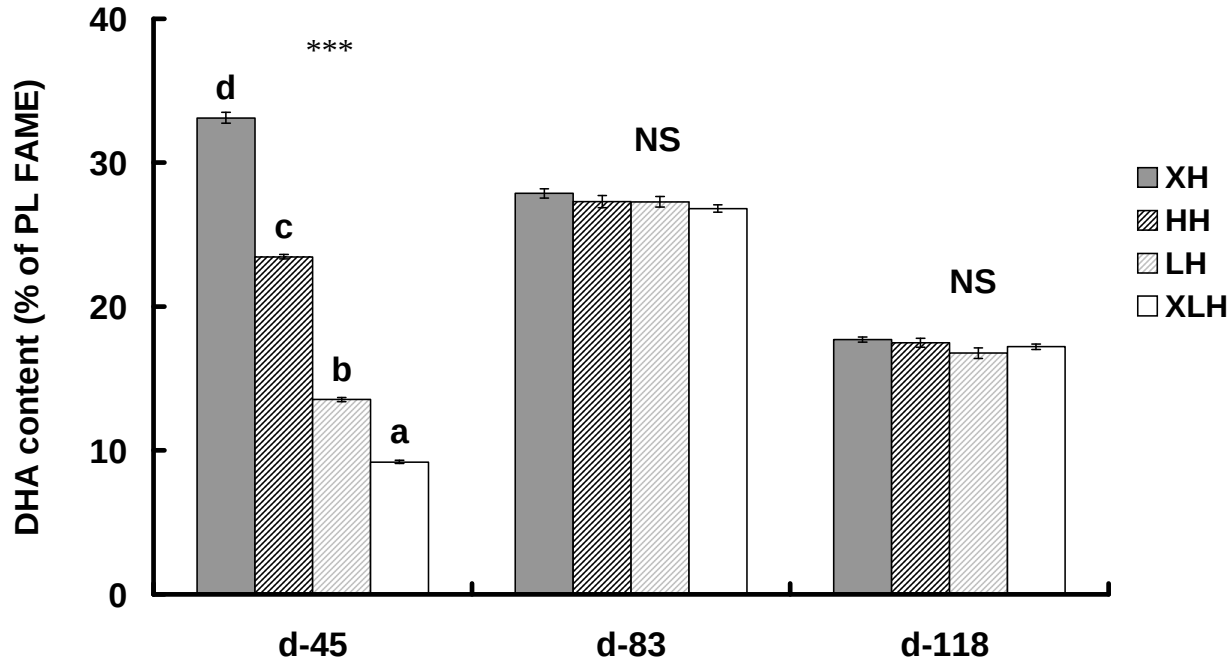

Fig.7. DHA content in PL of d-45 larvae (n=4), d-83 (n=4) and d-118 juveniles fed the R-diet (n=4), according to the larval initial diet. Statistical effect of initial diet is indicated (NS non significant, ***P<0.001) for each date. [a, b, c, d] differing letters denote significant difference at P<0.05 for each date.

Table 1 ; Vagner *et al.*

Table 1. Formulation (g.100 g^{-1}), chemical composition (% DM) and fatty acid composition in TL (% FAME) of the four experimental larval diets (XH, HH, LH and XLH) used in the larval experiment (exp.1).

Ingredients [†]	XH	HH	LH	XLH
Fish meal LT 94	11	11	11	11
Defatted fish meal	41	41	41	41
CPSP 90	11	11	11	11
Soy oil	0	0	1	1
Soy lecithin	7	16	21	23
Marine lecithin LC 40	19	9	2	0
Vitamin mixture [††]	7	7	7	7
Mineral mixture [§]	3	3	3	3
Betaine	1	1	2	2
Cellulose	1	1	1	1
Chemical composition				
Dry matter (%)	91.4	91.0	90.3	90.7
Crude protein (% DM)	63.9	59.2	57.0	57.1
Crude fat (% DM)	18.5	19.1	19.9	20.2
Ash (% DM)	13.9	13.9	14.0	13.9
HUFA n-3 (% DM)	3.8	1.8	0.8	0.5
EPA+DHA (% DM)	3.7	1.7	0.7	0.5
Fatty acids composition in TL				
18:2n-6	18.6	35.2	44.6	47.2
18:3n-6	0.1	0.3	0.1	0.2
20:4n-6	1.7	0.8	0.5	0.2
18:3n-3	1.9	3.3	4.1	4.4
20:5n-3	9.1	4.7	2.2	1.7
22:6n-3	20.5	9.8	3.9	2.2
Σ saturated	27.5	26.1	24.9	24.1
Σ mono-unsaturated	18.5	18.1	18.7	18.7
Σ n-6	20.8	36.7	45.4	48.0
Σ n-3	32.9	19.0	11.0	9.2

[†] Sources: fish meal LT 94: Norse (Fyllingsdalen, Norway); hydrolysed fish meal: Archimex (Vannes, France); fish protein hydrolysate CPSP 90: Sopropêche (Boulogne sur mer, France); soy oil: Système U (Créteil, France); soy lecithin: Louis François (Saint-Maur, France); marine lecithin LC 60: Phosphotech (Saint-Herblain, France).

[††] Vitamin mixture (g kg^{-1} vitamin mix): retinyl acetate, 1; cholecalciferol, 2.5; DL-α-tocopheryl acetate, 5; menadione, 1; thiamine-HCL, 0.1; riboflavin, 0.4; D-calcium panththenate, 2; pyridoxine-HCL, 0.3; cyanocobalamin, 1; niacin, 1; choline, 200; ascorbic acid (ascorbyl polyphosphate), 5; folic acid, 0.1; D-biotin, 1; meso-inositol, 30.

[§] Mineral mixture (g kg^{-1} mineral mix): KCL, 90; KI, 0.04; $CaHPO_4$ $2H_2O$, 500; NaCl, 40; $CuSO_4$ $5H_2O$, 3; $ZnSO_4$ $7H_2O$, 4; $CoSO_4$, 0.02; $FeSO_4$ $7H_2O$, 20; $MnSO_4$ H_2O, 3; $CaCo_3$, 215; MgOH, 124; Na_2SeO_3, 0.03; NaF, 1.

Table 2; Vagner et al.

Table 2. Formulation (g.100 g^{-1}), chemical composition (%DM) and fatty acid composition in total lipid (% fatty acid methyl esters FAME) of the experimental HUFA-restricted diet (R-diet) and the HUFA-control diet (C-diet) used in the juvenile experiment (exp. 2).

Ingredients †	R-diet	C-diet
Lupin without pellicle	50	50
Fish meal LT 94	12	12
Defatted fish meal	8	8
Wheat amygluten 110	7	7
Fish hydrolysate CPSP 90	8	8
Vitamin mixture ††	1	1
Mineral mixture §	1	1
Betaine	0.5	0.5
Methionine	0.2	0.2
Precooked starch	3.7	3.7
Soy lecithin	2	2
Rapeseed oil	6.6	0
Cod-liver oil	0	6.6
Chemical composition		
Dry matter (%)	92.2	92.0
Crude protein (% DM)	51.8	52.2
Crude fat (% DM)	14.9	15.6
Ash (% DM)	6.5	6.5
n-3 HUFA (% DM)	0.4	1.6
EPA+DHA (% DM)	0.3	1.4
Fatty acids composition in TL		
18:2n-6	20.2	11.3
18:3n-6	0.1	0.1
20:4n-6	0.1	0.3
18:3n-3	8.5	4.3
20:5n-3	1.2	4.8
22:6n-3	1.6	6.1
Σ saturated	13.2	17.8
Σ mono-unsaturated	54.3	52.5
Σ n-6	20.7	12.3
Σ n-3	11.9	17.5

† Sources: lupin without pellicle: Le Gouessant® aquaculture (Lamballe, France); fish meal LT 94: Norse (Fyllingsdalen, Norway); wheat amygluten 110: Chamtor Vitalor (Bazancourt, France); fish protein hydrolysate CPSP 90: Sopropêche (Boulogne sur mer, France); precooked starch: Prégéflo Roquette frères (Lestrem, France).

†† Vitamin mixture (g kg^{-1} vitamin mix): retinyl acetate, 1; cholecalciferol, 2.5; DL-α-tocopheryl acetate, 5; menadione, 1; thiamin-HCL, 0.1; riboflavin, 0.4; D-calcium panththenate, 2; pyridoxine-HCL, 0.3; cyanocobalamin, 1; niacin, 1; choline, 200; ascorbic acid (ascorbyl polyphosphate), 5; folic acid, 0.1; D-biotin, 1; meso-inositol, 30.

§ Mineral mixture (g kg^{-1} mineral mix): KCL, 90; KI, 0.04; $CaHPO_4$ $2H_2O$, 500; NaCl, 40; $CuSO_4$ $5H_2O$, 3; $ZnSO_4$ $7H_2O$, 4; $CoSO_4$, 0.02; $FeSO_4$ $7H_2O$, 20; $MnSO_4$ H_2O, 3; $CaCo_3$, 215; MgOH, 124; Na_2SeO_3, 0.03; NaF, 1.

Table 3 ; Vagner *et al.*

Table 3. Total lipid content (TL in % wet weight WW), polar lipid (PL in % TL) and FA profiles (in % FAME) of neutral lipids (NL) and PL in d-45 larvae. Values are mean ± SE (n=4). Statistical significance of diet is indicated (NS no significant; * $P<0.05$, ** $P<0.01$ and *** $P<0.001$). Values not sharing a common letter in the same line are significantly different.

	D-45 larval composition								Statistical analysis
	XH*1*		HH*1*		LH*1*		XLH*1*		
	mean	SE	mean	SE	mean	SE	mean	SE	
TL (% WW)	2.3	0.2	2.9	0.2	2.9	0.2	2.7	0.2	NS
PL (% TL)	47 [a]	4	44 [ab]	2	42 [b]	0	41 [b]	1	*
NL									
16:0	18.9 [a]	0.3	19.2 [a]	0.2	17.9 [b]	0.2	17.4 [b]	0.4	***
18:0	4.4 [a]	0.1	3.9 [b]	0.2	4.4 [a]	0.2	4.6 [a]	0.2	***
18:1	0.2 [a]	0.1	0.2 [ab]	0.2	0.1 [b]	0.1	0.0 [b]	0.1	*
18:2n-6	18.7 [a]	0.1	36.0 [b]	0.1	43.4 [c]	0.2	44.4 [d]	0.3	***
18:3n-6	0.2 [a]	0.0	0.1 [b]	0.0	0.5 [c]	0.0	0.9 [d]	0.1	**
20:2n-6	1.2 [a]	0.0	1.4 [b]	0.1	1.3 [ab]	0.0	1.2 [a]	0.1	*
20:4n-6	1.4 [a]	0.0	0.7 [b]	0.0	0.3 [c]	0.0	0.2 [d]	0.0	**
18:3n-3	1.8 [a]	0.0	3.1 [b]	0.0	3.7 [c]	0.0	3.8 [c]	0.1	***
18:4n-3	0.5 [a]	0.0	0.4 [ab]	0.0	0.4 [ab]	0.0	0.4 [b]	0.0	*
20:4n-3	0.4 [a]	0.0	0.2 [b]	0.0	0.2 [b]	0.0	0.2 [b]	0.0	***
20:5n-3	7.7 [a]	0.1	3.9 [b]	0.1	1.8 [c]	0.1	1.1 [d]	0.0	***
22:5n-3	0.7 [a]	0.0	0.5 [b]	0.0	0.3 [c]	0.0	0.3 [c]	0.0	***
22:6n-3	18.2 [a]	0.2	7.3 [b]	0.2	2.3 [c]	0.0	1.4 [d]	0.0	***
Σ saturated	26.8 [a]	0.2	25.4 [b]	0.2	24.6 [c]	0.1	24.4 [c]	0.1	***
Σ MUFAs	22.2 [a]	0.1	20.7 [c]	0.3	21.1 [bc]	0.0	21.4 [b]	0.0	***
Σ PUFAs	51.0 [a]	0.4	53.9 [b]	0.3	54.3 [b]	0.3	54.1 [b]	0.3	***
PL									
16:0	22.6 [a]	0.2	21.6 [b]	0.2	20.1 [c]	0.4	19.8 [c]	0.3	***
18:0	6.2 [a]	0.3	6.6 [b]	0.1	7.2 [c]	0.2	7.5 [c]	0.1	***
18:1	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.1	NS
18:2n-6	8.8 [a]	0.1	22.6 [b]	0.1	34.9 [c]	0.2	39.6 [d]	0.2	***
18:3n-6	0.0 [a]	0.0	0.2 [b]	0.1	0.3 [c]	0.1	0.5 [c]	0.0	***
20:2n-6	1.3 [a]	0.1	1.7 [b]	0.1	1.8 [b]	0.0	1.9 [b]	0.0	***
20:3n-6	0.1 [a]	0.0	0.1 [a]	0.0	0.2 [b]	0.0	0.2 [b]	0.0	*
20:4n-6	3.1 [a]	0.0	1.9 [b]	0.0	1.0 [c]	0.0	0.6 [d]	0.1	***
18:3n-3	0.5 [a]	0.0	1.2 [b]	0.0	1.7 [c]	0.0	1.9 [d]	0.0	***
20:5n-3	9.7 [a]	0.1	6.8 [b]	0.0	4.7 [c]	0.1	3.7 [d]	0.1	***
22:5n-3	0.5 [a]	0.0	0.6 [ab]	0.0	0.6 [b]	0.0	0.6 [b]	0.0	*
22:6n-3	33.1 [a]	0.4	23.5 [b]	0.2	13.5 [c]	0.2	9.2 [d]	0.1	***
Σ saturated	29.8 [a]	0.3	29.1 [a]	0.1	28.2 [b]	0.3	28.1 [b]	0.2	**
Σ MUFAs	12.7 [ab]	0.3	12.1 [a]	0.3	12.6 [ab]	0.1	13.0 [b]	0.0	*
Σ PUFAs	57.6 [a]	0.7	58.8 [b]	0.3	59.2 [b]	0.4	58.9 [b]	0.3	*

Table 4 ; Vagner *et al.*

Table 4. Total lipid content (TL in % wet weight WW), polar lipid (PL in % TL) and FA profiles (in % FAME) of neutral lipids (NL) and PL in each treatment of d-118 R-groups. Values are mean ± SE (n=4). Statistical significance of diet is indicated (NS no significant; * $P<0.05$). Values not sharing a common letter in the same line are significantly different.

	D-118 juveniles								Statistical analysis
	XH2_R		HH2_R		LH2_R		XLH2_R		
	mean	SE	mean	SE	mean	SE	mean	SE	
TL (%WW)	9.8	0.6	8.8	0.2	8.8	0.5	9.3	0.2	NS
PL (% TL)	13.6	1.0	14.2	0.8	11.6	0.9	14.1	1.0	NS
NL									
16:0	11.4	0.1	11.8	0.1	11.4	0.1	11.9	0.1	NS
18:0	2.9	0.0	3.0	0.0	2.9	0.0	3.0	0.0	NS
18:1	0.1	0.0	0.1	0.0	0.1	0.1	0.1	0.0	NS
18:2n-6	16.2	0.2	16.6	0.3	17.0	0.1	16.8	0.1	NS
18:3n-6	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	NS
20:2n-6	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0	NS
20:3n-6	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	NS
20:4n-6	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	NS
18:3n-3	6.2	0.1	6.3	0.2	6.5	0.0	6.4	0.0	NS
18:4n-3	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0	NS
20:3n-3	0.1 [a]	0.0	0.1 [b]	0.0	0.1 [b]	0.0	0.1 [b]	0.0	**
20:4n-3	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	NS
20:5n-3	2.4 [a]	0.0	2.1 [b]	0.1	2.1 [b]	0.1	2.0 [b]	0.1	*
22:5n-3	0.5 [a]	0.0	0.4 [ab]	0.0	0.4 [b]	0.0	0.4 [b]	0.0	*
22:6n-3	2.8 [a]	0.1	2.3 [b]	0.1	2.2 [b]	0.1	2.3 [b]	0.1	*
Σ saturated	17.7	0.2	17.6	0.4	17.1	0.1	17.2	0.2	NS
Σ MUFAs	51.9	0.2	52.7	0.3	52.7	0.2	52.8	0.2	NS
Σ PUFAs	30.3	0.2	29.7	0.3	30.2	0.2	30.0	0.2	NS
PL									
16 :0	15.4	0.1	15.5	0.2	15.3	0.1	15.6	0.1	NS
18 :0	7.2	0.1	7.4	0.1	7.2	0.1	7.2	0.0	NS
18 :1	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	NS
18:2n-6	13.1	0.2	13.3	0.2	13.4	0.3	13.5	0.2	NS
18:3n-6	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	NS
20:2n-6	0.9	0.0	1.0	0.0	1.0	0.0	1.0	0.1	NS
20:3n-6	0.3	0.0	0.3	0.0	0.3	0.0	0.2	0.0	NS
20:4n-6	1.5	0.0	1.5	0.0	1.5	0.0	1.5	0.0	NS
18:3n-3	3.4	0.1	3.5	0.1	3.4	0.1	3.5	0.1	NS
18:4n-3	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	NS
20:3n-3	0.1	0.0	0.1	0.0	0.2	0.0	0.1	0.0	NS
20:4n-3	0.2 [a]	0.0	0.2 [a]	0.0	0.3 [b]	0.0	0.2 [a]	0.0	*
20:5n-3	6.6	0.1	6.5	0.2	6.4	0.1	6.5	0.1	NS
22:5n-3	1.1	0.0	1.1	0.0	1.1	0.0	1.1	0.0	NS
22:6n-3	17.7	0.2	17.5	0.4	16.8	0.6	17.2	0.2	NS
Σ saturated	24.6	0.2	24.8	0.3	24.7	0.2	24.7	0.3	NS
Σ MUFAs	29.8	0.1	29.7	0.3	30.3	0.3	29.7	0.1	NS
Σ PUFAs	45.6	0.1	45.5	0.4	45.0	0.4	45.6	0.2	NS