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Abstract:

Two experiments were conducted to test if manipulations of the Arginine-Nitric oxide pathway during the early life of rainbow trout would act on its early myogenic process. In experiment 1, trout embryos were immersed at 72 degree days post-fertilization (°dpf) or 104°dpf in water alone (control treatment, C) or containing 2mM/L L-Arg (treatment A) or 1mM/L of L-NAME, a NOS inhibitor (treatment N). We observed the beginning of expression of myf5 and fmhc genes at 72°dpf and 96°dpf, respectively. "A" treatment doubled the free Arg content of eggs but did not affect either the pattern of expression of myf5 and fmhc, nor white muscle cross-sectional area and number of white muscle fibres at hatching, nor embryo survival and fry growth. "N" treatment also did not affect these markers. In experiment 2, trout fry were fed from first feeding onwards and during 20 days either a control diet (C) or the same diet supplemented with L-NAME (0.1g/100g diet, N-diet). In C-fed fry, distribution of a single meal after overnight fasting induced changes in pcna, myod1, myog, fmhc, inos, nnos and ctsd gene expressions. N-feeding decreased fry growth but did not change their growth trajectory or survival. Twenty days of N-feeding led, compared to C-feeding, to changes in kinetics of transcription of pcna, myod1, myog, fmhc, inos, nnos, ctsd genes and to decreased white muscle cross-sectional area, total number of white muscle fibres, and number of large muscle fibres. L-NAME feeding thus decreased fry muscle growth by altering both hyperplasia and hypertrophy.

Keywords:

Fish nutrition; Arginine; L-NAME; Myogenesis; Gene expression; Kinetic; Hyperplasia; Hypertrophy.

Highlights of the manuscript:

- Myf5 and fmhc gene expressions were evidenced at 72°dpf and 96°dpf, respectively
- Embryonic arginine or L-NAME pulses do not affect muscle fibre number at hatching
- Feeding induces changes in transcription of myogenic and muscle growth-related genes
- L-NAME feeding alters the kinetics of transcription of muscle growth-related genes
- L-NAME feeding decreases muscle growth, fibre recruitment and fibre hypertrophy

Statement of relevance

Knowledge on the effects of arginine on fish myogenesis will help maximising fish muscle growth

Abbreviations:

Arg, arginine; Ctsd, cathepsin D; d, diameter of white muscle fibres; dpf, days post fertilization; °dpf, degree days post fertilization; fMHC, fast myosin heavy chain; IAA, indispensable amino acid; iNOS, inducible nitric oxide synthase; ISH, *in situ* hybridization; L-NAME, N(ω)-nitro-L-arginine methyl ester; MRF, myogenic regulatory factor; MRF4/Myf6, myogenic factor 6; Myf5, myogenic factor 5; MyoD1, myoblast determination protein 1; Myog, myogenin; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PCNA, proliferating cell nuclear antigen; TCSAWM, total cross-sectional area of white muscle; TNWF, total number of white muscle fibres.

1. Introduction

Teleosts which reach a large adult body size, such as the rainbow trout (*Oncorhynchus mykiss*), present the unique feature of producing new muscle fibres during a large part of the life cycle. After the initial two muscle layers (deep fast and superficial slow) have been formed from lateral and adaxial cells, increase in muscle fibre number (hyperplastic growth of muscle) continues in two phases; stratified hyperplasia generates new fibres along a distinct germinal layer, while during mosaic hyperplasia, new fibre production is disseminated across the whole myotome (Rowlerson and Veggetti, 2001). The time window of stratified and mosaic hyperplasia varies between fish species. In rainbow trout, both stratified hyperplasia and mosaic hyperplasia begin early, before hatching (Xi et al., 2001). Hyperplasia is the major process for the increase in muscle cross sectional area in rainbow trout fry, but, during growth, its relative importance decreases at the expense of hypertrophy (increase in size of existing fibres) (Stickland, 1983). These developmental-related changes in muscle cellular phenotype result from temporal changes in expression levels of genes regulating specific transition points in myogenesis, the myogenic regulatory factors (MRFs). Myoblast determination protein (MyoD), myogenic factor 5 (Myf5) and myogenic regulatory factor 6 (Myf6/MRF4) function as myogenic determination factors, while myogenin (Myog) acts as a differentiation factor, as do MRF4 and MyoD; myogenic progenitor cells can either activate Myf5 and MyoD and differentiate or proliferate, providing a reserve cell population for muscle growth during development (Buckingham and Rigby, 2014).

Although most of the knowledge on the nutritional regulation of muscle growth in fish are based on studies undertaken with juveniles, different studies had shown that early nutrition can have a profound effect on muscle growth. Changes in first-feed protein source (Alami-Durante et al., 1997; Ostaszewska et al., 2008), protein/energy ratio (Alami-Durante

et al., 2014), protein level (Saavedra et al., 2016) and degree of hydrolysis of protein sources (Canada et al., 2018) were successively proved to modify the pattern of early muscle growth at cellular or/and molecular levels. Other studies, with first-feeds varying in both protein sources and level of incorporation of several indispensable amino acids (IAA) (Canada et al., 2016; Saavedra et al., 2016) showed that changes in IAA profile had consequences on muscle cellularity and expression of muscle-growth related genes. The knowledge on the specific role that each IAA might play *in vivo* during fish early muscle growth is scarce and limited to lysine, which was found to alter the distribution of white fibre diameters in Nile tilapia larvae albeit without any effect on fibre number and proliferating nuclei (Aguiar et al., 2005), to phenylalanine, which also act on distribution of white muscle fibres in Nile tilapia larvae (Yamashiro et al., 2016), and to methionine, which regulates the gene expression of myogenin and fast myosin heavy chain in rainbow trout fry (Fontagné-Dicharry et al., 2017). The role of other IAA on early myogenesis and muscle growth of fish remains to our knowledge to be investigated.

Arginine (Arg) is an IAA in fish (NRC, 2011). Without metabolic processing, Arg is reported to be a potent secretagogue of several pancreatic hormones in fish (Plisetskaya et al. 1991; Mommsen, 2001) but data suggested that changes in plasma insulin level occurred only when fish are fed dietary arginine supplementation far exceeding the requirement levels (Lall et al., 1994). Arg metabolism leads to the formation of γ-guanidinoacetate and then creatine and creatine phosphate, which are essential for muscle function. Arg is also a precursor of ornithine, glutamine, glutamate and proline, required for the de novo synthesis of protein, and of putrescine, essential to the synthesis of polyamines which stimulate cell growth and proliferation (Miller-Fleming et al., 2015). Arginine is also degraded by nitric oxide synthases (NOS) into citruline and nitric oxide (NO), which regulates different skeletal muscle functions such as force production, blood flow, respiration and glucose homeostasis (Stamler

and Meissner, 2001). It was demonstrated *in vitro*, by using NO donors (L-Arg, DETA-NO, SNP, SNAP) and/or pharmacological inhibitors of NOS activity (L-NAME, NMMA), that NO also mediates the activation (Anderson and Pilipowicz, 2002, Anderson and Wozniak, 2004; Betters et al., 2008; Tatsumi et al., 2002a,b, Wozniak and Anderson, 2007) and the proliferation of muscle precursor cells (Buono et al., 2012; Long et al., 2006; Ulibarri et al., 1999), as well as the fusion and differentiation of myoblasts (Lee et al., 1994; Long et al., 2006; Li et al., 2016) in mammals and birds. *In vivo*, a decreased NO level leads to decreased activation of muscle precursor cells (Anderson et al., 2000; Tatsumi et al., 2006) as shown in mice and rats injected intraperitoneally with L-NAME, to decreased mean muscle fibre diameter (Wang et al., 2001) and decreased myonuclear addition to mature muscle fibres (Gordon et al., 2007) as shown in rats fed with L-NAME in drinking water, and to decreased expression of myogenin and myosin (Carrazo et al., 2014) as shown in chick injected *in ovo* with NPLA (a NOS inhibitor). Arg supplementation in birds led to increased mean myofibre diameter (Fernandes et al., 2009) as shown in chick fed started diets supplemented with Arg, and to increased expression of myod and myogenin (Subramaniyan et al., 2019) as shown in chicken hatched from eggs injected with Arg. In mammals, Arg supplementation led to increased formation of primary muscle fibres, to stimulation of myogenic proliferation and to delay in muscular differentiation (Bérard and Bee, 2010; Kalbe et al., 2013) as shown in foetuses from gilts fed diets supplemented with Arg during early gestation, and to increased number of muscle fibres (Shi et al., 2018) as shown in growing-finishing pigs from sows fed diets supplemented with Arg during early gestation. Studies on the effect of Arg supplementation on fish muscle development are scarce. Available data only indicate that, *in vivo*, dietary Arg supplementation modified the frequency distribution of muscle fibres in juvenile Nile Tilapia (Neu et al., 2016) and had no effect on fibre density in juvenile Atlantic salmon (Østbye et al., 2018). *In vitro*, supplementing with the NO-donor ISDN to the culture

139 medium of single muscle fibre cultures from adult zebrafish led to increased activation of the
140 satellite cells lying alongside them (Zhang and Anderson, 2014), and supplementing with Arg
141 the culture medium of satellite cells extracted from juvenile Atlantic salmon led to increased
142 myogenin and myosin light chain expressions (Østbye et al., 2018).

143 In light of the importance of NO in the early myogenesis of mammals and birds, we
144 hypothesized that manipulation of the Arginine-NO pathway during the early life of fish
145 would also act on their early myogenic process with, given the importance of the number of
146 muscle fibres formed during early life for subsequent fish growth potential (e.g. Alami-
147 Durante et al., 2007; Macqueen et al., 2008), potential long-term consequences. We thus
148 studied the effects of *in vivo* early supplementations (during embryogenesis or at first feeding)
149 with L-Arg or L-NAME on molecular and cellular markers of muscle growth mechanisms
150 (hyperplasia and hypertrophy), with rainbow trout as model.

151

152 **2. Materials and methods**

153

154 The experiments were carried out with a certificate for experimenting on live animals
155 (N°B6410006) and in accordance with the EU Directive for the protection of animals used for
156 scientific purposes (N°2010/63/EU) and the French Decree for animal experimentation
157 (N°2013-118).

158

159 *2.1. Experiment 1 on rainbow trout embryos*

160

161 Diploid eggs were obtained in November from the INRA rainbow trout strain
162 maintained in the INRA experimental fish farm of Lees Athas (agreement N°A64-104-1) and
163 randomly distributed into 32 small mesh racks (10 x 10 cm, density = 500 eggs per rack)

themselves randomly disposed in 2 troughs in a flow-through system supplied with spring water (temperature = 8.0 ± 0.3°C). As shown on the schematic diagram of the trial (figure 1), two racks were used to finely characterize somitogenesis in the rainbow trout strain used. For this, 10 embryos (5 x 2 racks) were sampled each day at 9 am and 3 pm, from day 9 to day 24 post fertilization (dpf), i.e. from 72 to 192 degree days post-fertilization (°dpf), based on results indicating that somite formation can begin at 70 °dpf (Vernier, 1969) and last up to 175 °dpf (Ballard, 1973) in rainbow trout. These embryos were fixed in a mixture of 2.5% glutaraldehyde, 2.5% paraformaldehyde, 0.5% sucrose in 0.08M cacodylate buffer, pH 7.4 (Galloway et al., 1998) as we found in a preliminary experiment that this fixative kept the transparency of trout embryos and thus allowed to count the number of somite formed with accuracy. These embryos were then dehydrated and kept in methanol at +4°C until quantification of somitogenesis. The other 30 racks were used to characterize the effect of immersion of embryos in arginine or L-NAME baths on molecular and cellular markers of myogenesis and muscle growth. For this, the racks containing the embryos were carefully retrieved from the incubation troughs and immersed during 4 hours in aerated small tanks containing water alone as a control ("C" treatment), or water with diluted L-arginine (2mM/L, "A" treatment) or diluted L-NAME (1mM/L, "N" treatment). These treatments were performed in triplicate on half of the racks at 9 dpf (i.e. 72 °dpf) and on the other racks at 13 dpf (i.e. 104 °dpf). These two time-windows were chosen, based on literature data (Ballard, 1973; Vernier, 1969), in order to act before the formation of the first somite or during somitogenesis. As we aimed to modify embryonic myogenesis without altering survival, L-NAME bath concentration (1mM/L) was chosen based on a previous study showing that salmonid embryos and alevins react to this L-NAME concentration, as shown by their cardiovascular response, and that incubation in 1mM/L L-NAME affects neither egg hatchability nor appearance and behaviour of exposed alevins (Eddy et al., 1999). Arg bath

concentration (2mM/L) was chosen based on results showing that incubation of cultured mouse myofibres in 2mM/L Arg stimulated the activation of the satellite cells residing alongside them (Betters et al., 2008). In order to test if a balneation in 2mM Arg for 4 hours was efficient to increase Arg concentration in eggs, 80 eggs were sampled at the end of the balneation held at 13 dpf in the A and C racks. These eggs were frozen in liquid nitrogen and kept at -80°C until chemical analyses. For *in situ* hybridization (ISH) analyses, 10 embryos per rack having undergone the A, N or C treatment at 9 dpf were sampled at 10, 12, 14, 18 and 22 dpf, and 10 embryos per rack having undergone the A, N or C treatment bath at 13 dpf were sampled at 14, 18 and 22 dpf. These embryos were fixed for 24h with 4% PFA in PBS pH 7.4, rinsed in PBS, dehydrated in methanol baths and kept in methanol at -20°C until further analyses. Samples were subsequently taken at the end of hatching (48dpf, i.e. 384°dpf). 27 hatched fry were retrieved from each rack, euthanized by overdose of benzocaine (60 mg/L), and individually weighed; 20 of them were frozen in liquid nitrogen and stored at -80 °C for mRNA extraction and generation of molecular probes for *in situ* hybridization analyses; the remaining 7 fry were fixed in Serra's liquid (absolute ethanol 6V; 37% formalin 3V; glacial acetic acid 1V), dehydrated and preserved in butanol until histological analyses. In order to test if the embryonic treatments with C, A, or N bath at 9 or 13 dph had consequences on post hatch body growth, the fry from the triplicates of each embryonic treatment were pooled at the end of hatching. They were then distributed in new triplicates groups of 100 fry in independent circular tanks (50 L) in a flow-through system supplied with spring water (temperature = 8.0 ± 0.3°C) and fed with a commercial feed (Bio optimal start, Biomar) from first feeding onwards (73 dpf, i.e. 584°dpf). The fry were hand-fed 6 to 8 times per day (from 9 am to 5 pm) to apparent satiation, with pellets increasing in size over time (200-500 µm for 2 weeks, 500-800 µm for 3 weeks, and 0.8-1.1 mm thereafter). Fry were group (tank) weighed every 3 weeks to follow growth.

214

215 *2.2. Experiment 2 on first-feeding rainbow trout fry*

216

217 Diploid rainbow trout eggs were obtained in April from a commercial fish farm
218 (Viviers de France) and incubated in the INRA experimental fish farm of Lees Athas in spring
219 water (temperature of 8.0 ± 0.1°C) until hatching (366°dpf). Hatched fry were maintained
220 under these conditions until 495°dpf and then transferred to the INRA experimental fish farm
221 of Donzacq (agreement N°A40-228-1) and distributed into 6 independent circular tanks (50
222 L) in a flow-through system supplied with spring water (temperature of 17 ± 1°C) under
223 natural photoperiod (May-June). At 546°dpf (day 0 of the feeding trial), the emerging fry,
224 still having small yolk reserves, were fed with two diets formulated with plant protein sources
225 (Table 1) for 20 days (triplicate tanks per diet; 100 fry per tank). The control diet (C),
226 formulated to cover the nutritional requirements of rainbow trout (NRC, 2011), was
227 manufactured without oil using a twin-screw extruder (BC 45, Clextral, France) and ground in
228 fractions of 200-500µ and 500-800µm. Both fractions were then divided in two parts. The
229 first part was coated with fish oil to obtain diet C, while the second part was coated with N
230 (ω)-nitro-L-arginine methyl ester (L-NAME, 0.1g/100g diet) mixed with fish oil to obtain diet
231 N. The fry were hand-fed 6 to 8 times per day (from 9 am to 5 pm) to apparent satiation, with
232 diet particle fractions of 200-500µm during the first 15 days and 500-800µm later. The
233 amount of feed distributed was recorded weekly, and mortality daily. On day 20 of the
234 feeding trial, overnight unfed fry (16h food deprived) were sampled at 9 am (n=9 per diet),
235 just before distribution of an ad libitum single meal to the remaining fry. To study the post-
236 prandial expression of myogenic and muscle growth-related genes, fry were sampled at 1 h, 2
237 h, 3 h, 5 h, 7 h and 9 h after the meal. In order to limit handling stress in successive
238 samplings, the required number of fry (n=9 per diet x 7 sampling time) was withdrawn from

one of the three tanks at each sampling time. These fry were euthanized by an overdose of benzocaine (60 mg/L), weighed and measured, frozen in liquid nitrogen, and stored at -80 °C until molecular analysis. For analysis of muscle cellularity, 9 fry per diet (3 per tank) were additionally sampled, euthanized by an overdose of benzocaine, weighed and measured, fixed in Serra's liquid (absolute ethanol 6V; 37% formalin 3V; glacial acetic acid 1V), and dehydrated until further histological analysis.

2.3. Analysis

2.3.1. Somitogenesis and egg free Arg content

The embryos kept in ethanol were dechorionated with fine forceps and the number of somites formed counted from 9 to 24 dpf using a binocular microscope. Free Arg content was quantified using K-Lage kit (Megazyme, Ireland) on frozen eggs sampled after the 13-dpf bath.

2.3.2. Molecular analysis

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen samples using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and stored in Nuclease-free water at – 20°C. Samples were subjected to electrophoresis on 1% agarose gels to confirm the integrity of the 28S and 18S rRNA bands, and RNA purity was assessed by the 260/280 nm absorbance ratio. Thereafter, 2 µg of total RNA was reverse transcribed using 200 U SuperScript™ III Reverse Transcriptase (Invitrogen) and 500 ng of random primers (Promega) in a total volume of

20 µL (25°C for 10 min, 55°C for 1 h, 70°C for 15 min). Myf5 (Myogenic factor 5), myod1 (myoblast determination protein 1), myog (myogenin), pcna (proliferative cell nuclear antigen), fmhc (fast myosin heavy chain), inos (inducible nitric oxide synthase) and ctsd (cathepsin D) cDNA fragments were obtained by RT-PCR using previously published primers (Alami-Durante et al., 2010, 2014; Chauvigné et al., 2003; McNeill and Perry 2005; Salem et al., 2006). Primers for neuronal nitric oxide synthase (nNOS; F: gccatccttcgagtatcagg; R: cttgagactttccggtctcg) were designed for experiment 2 using GeneBank sequence DQ640498 and primer 3 software (University of Massachusetts). All primers were synthesised by Eurogentec (Belgium) and amplicons confirmed by sequencing (Eurofins Genomics, Ebersberg, Germany). For real-time RT-PCR assays, the Roche Lightcycler 480 system was used (Roche Diagnostics, Neuilly-sur-Seine, France). The assays were performed using a reaction mix of 6 µL per sample, each of which contained 2 µL of diluted (dilution=35) cDNA template, 0.12 µL of each primer (10 µmol L^{-1}), 3 µL iQ™ SYBR ® Green Supermix (Bio-Rad) and 0.76 µL DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany). The PCR protocol was initiated at 95°C for 10 min for initial denaturation of the cDNA and hot-start DNA polymerase activation and continued with 40 cycles of a two-step amplification program (15 s at 95°C; 30 s at 60°C). Melting curves were systematically monitored (temperature gradient at 0.5°C 10 s^{-1} from 55 to 94°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each real-time RT-PCR run included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (reverse transcriptase- and cDNA template-free samples). Standard curves, consisting of five serial dilutions in triplicate of a pool of cDNAs, were obtained for each cDNA template by plotting CT values against the $\log_{10}$ of the different dilutions. Real-time PCR efficiency (E) was calculated from standard curves according to Pfaffl (2001).

289 *Whole mount in situ hybridization*

290 *In situ* hybridization (ISH) was performed in order to follow early (commitment) and
291 late (terminal differentiation) stages of myogenesis, marked by myf5 and fmhc expressions,
292 respectively. The myf5 and fmhc cDNA fragments obtained by RT-PCR were cloned into the
293 pCR®II-TOPO®vector (TOPO TA Cloning kit, Invitrogen) according to the manufacturer's
294 protocol. After appropriate linearization of the plasmids, sense and anti-sense RNA probes
295 were generated using SP6 and T7 polymerases in the presence of digoxigenin-11UTP (DIG
296 RNA labelling kit, Roche). Embryos sampled in C, A, and N-tanks were, following
297 rehydration in graded methanol/PBS baths, dechorionated with fine forceps in PBS and
298 processed according to established procedures with minor modifications. Embryos were
299 incubated in PBST containing 50µg/ml proteinase K (Roche) for 15 min, washed in PBST,
300 refixed in a 4% PFA-0.2% glutaraldehyde solution for 20 min, and washed in PBST. Embryos
301 were then prehybridized for 2 h at 65°C in a solution containing 50% formamide, 5x SSC, 0.1
302 mg/ml yeast transfer RNA (Sigma), 0.1 mg/ml heparin and 0.1% Tween20. Dig-riboprobes
303 were denatured for 5 min at 65°C, diluted in hybridization buffer (0.5-1µg/ml) and incubated
304 with the embryos overnight at 65°C. Negative controls with sense probe and without probe
305 were performed. Embryos were then washed in 2x SSC baths to remove aspecific hybrids. To
306 detect hybridized probes embryos were blocked for 90 min in PBST containing 2% sheep
307 serum and 0.2% triton (blocking solution), and then incubated overnight at 4°C in 1:4000
308 diluted alkaline phosphatase conjugated anti digoxigenin (Roche) in blocking solution. The
309 staining was performed overnight in a dark chamber with a solution containing Levamisol and
310 NBT-BCIP (Boehringer Mannheim). Colour development was stopped in H_2O for 10min, 4%
311 PFA for 10 min, and PBS for 30 min.

312

313 *Quantification of mRNA relative abundances*

314 The pre- and post-prandial gene expression data obtained from qPCR were analysed
315 using the ΔCT method (Pfaffl, 2001) with the C-fed fry at time 0h as a control. Data were
316 then normalized using the NORMA-gene algorithm which uses a least squares method to
317 minimize data variability and which does not require the identification and validation of
318 reference genes (Heckmann et al., 2011), as yet validated in trout (Borey et al., 2016).
319
320 *2.3.3. Quantitative histological analyses*
321
322 The tails of the fry kept in butanol were embedded in paraffin and cut transversely into
323 sections (10 µm thick) that were stained with haematoxylin and orange G (Gabe, 1968).
324 Cellular analyses were performed on a dorsal quadrant of white myotomal muscle, as
325 previously defined (Alami-Durante et al. 1997). Muscle cellularity was quantified on one
326 section per fish, located at the vent level. Measurements were performed with Image-Pro Plus
327 software (Media Cybernetics). In experiment 1, the total cross-sectional area of one dorsal
328 quadrant of white muscle (TCSAWM) was measured and the total number of white muscle
329 fibres (TNWF) present in the quadrant was counted. In experiment 2, the individual area of all
330 the fibres present the dorsal quadrant of white muscle was additionally measured. The white
331 fibre individual equivalent area diameter (diameter of a circle whose area is the same as that
332 of the muscle fibre; will be referred to as ‘‘fibre diameter’’) was then calculated.
333
334 *2.3.4. Chemical analyses of diets*
335
336 The chemical composition of C-diet (Table 2) was analysed using the following
337 procedures: dry matter after drying at 105°C for 24h, protein content (N x 6.25) by the
338 Kjedhal method after acid digestion, lipid content by petroleum diethyl ether extraction

(Soxtherm), and gross energy after combustion in an adiabatic bomb calorimeter (IKA). The amino acid composition of the C-diet (Table 2) was analysed by HPLC by AgroBio laboratory (Vezin Le Coquet, France).

2.3.5. Statistical analyses

Statistical analyses were performed with R 3.2.3 Software (R Development Core Team, Vienna, Austria, www.R-project.org) and SigmaStat 3 computing program (SPSS, Chicago, Il, USA). Differences in survival, body weight and muscle cellularity were analysed, after appropriate transformation (arcsin, log, square root) to verify equality of variance and normality, by two-way anova (with "dpf" and "bath" as factors) followed by Newman-Keuls multiple range test to compare means when significant differences were found (exp. 1) or t-test (with "diet" as factor) (exp. 2). Differences in pre- and post-prandial expressions of myogenic and muscle growth-related genes were analysed in exp.2 by two-way anova (with "diet" and "hours after feeding" as factors) after rank-transformation when necessary followed by Newman-Keuls multiple range test to compare means when significant differences were found. Differences were considered significant when $P<0.05$.

3. Results

3.1. Experiment 1 on embryos

3.1.1. Somitogenesis

363 The first five somites were formed at 10 dpf (80°dpf). Somitogenesis then progressed
364 at a rate of about 5.7 somites formed per day, to reach 65 somites formed at 21 dpf (168°dpf)
365 (figure 2).
366
367 *3.1.2. Effect of A-bath on egg free Arg content*
368
369 The trout eggs immersed during 4h in the A-bath at 13dpf had, at the end of the bath, a
370 free Arg content twice higher (1.10 ±0.07 µM/g) than that of the eggs immersed in the C-bath
371 (0.52 ±0.09 µM/g) ($P < 0.0001$).
372
373 *3.1.3. Embryonic pattern of expression of myf5 and fmhc genes*
374
375 Myf5 expression began at 9 dpf (72°dpf) in the rostral domain of presomitic
376 mesoderm, on both sides of median axis (figure 3). At 10 dpf (80°dpf), myf5 was expressed in
377 all neoformed somites and presomitic mesoderm. At 14 dpf (112°dpf), i.e. when 22 somites
378 were formed, myf5 expression was weaker in the somites firstly formed at rostral level than in
379 somites lastly formed at caudal level, and the stronger myf5 expression was always noted in
380 the most caudal part of embryos in unsegmented mesoderm. At 18 dpf (144°dpf) i.e. when 50
381 somites were formed, myf5 transcripts accumulated preferentially in the lateral external
382 bordure and in the posterior region of formed somites; myf5 transcripts were always present
383 in quantity in unsegmented caudal mesoderm. At the end of somitogenesis, i.e. at 22dpf
384 (168°dpf), myf5 expression was localized in the posterior part and in the external lateral part
385 of all somites.
386 The first expression of fmhc was detected at 12dpf (96°dpf), i.e. when 16 somites were
387 formed (figure 4) and transcript location was limited to the most rostral trunk area (in about

388 10 somites). Observation of embryos in dorsal view showed that fmhc transcripts were
389 localized in the median part of rostral somites, on both sides of central structures. At 14 dpf
390 (112°dpf), i.e. when 28 somites were formed, the expression domain of fmhc spread out
391 laterally in the first formed somites and colonized the caudal region of embryos. From 18 dpf
392 (144°dpf) fmhc expression was very important and transcripts were localized in almost all
393 somite area.
394 Differences in the pattern of expression of myf5 and fmhc linked to embryonic
395 treatments (C-, A- or N-bath at 9 or 13 dpf) were not identified (figures 3,4).
396
397 *3.1.4. Survival, body weight, white muscle cross-sectional area, and total number of white*
398 *muscle fibres at hatching*
399
400 Survival at hatching was high (>93%) and not affected by bath at 9dpf or 13dpf in
401 2mM/L Arg or 1mM/L L-NAME (Table 3). Fry body weight at hatching was similar in the
402 different batches. Immersion of embryos in 2mM/L Arg or 1mM/L L-NAME at 9 dpf or 13
403 dpf affected neither the total cross-sectional area of white muscle nor the total number of
404 white muscle fibres at hatching (Table 3).
405
406 *3.1.5. Effect of embryonic treatments on post-hatch fry growth*
407
408 Immersion of embryos for 4h in 2mM/L Arg or 1mM/L L-NAME at 9 dpf or 13 dpf
409 had no effect on the subsequent post-hatch weight increase of fry (Figure 5).
410
411 *3.2. Experiment 2 on first feeding fry*
412

3.2.1. Fry survival and growth

The quantities of C- and N-diets distributed ad-libitum during the whole feeding trial were similar (14.5g/tank and 14.7g/tank, respectively). After 20 days of feeding, the survival of C-fed (96.4 ± 3.6%) and N-fed (93.4 ± 5.5%) fry was high and not different ($P=0.462$). The body weight ($P<0.0001$) and the total length ($P<0.0001$) of the C-fed fry were higher than those of N-fed fry (Figure 6), but the slopes ($P=0.589$) and the intercepts ($P=0.580$) of the length-weight relationships of C- and N-fed fry were similar (Figure 7).

3.2.2. Pre- and post-prandial expressions of genes involved in myogenesis, cell proliferation, muscle structure, arginine catabolism, and proteolysis

In C-fed fry, alterations in gene expressions occurred rapidly after the distribution of the single meal (Figure 8), with decreased transcript levels of pcna and fmhc at 1h. A similar trend, although not significant, was observed for myod1, myog, inos, nnos and ctsd (Figures 8,9). The transcript level of all the genes then increased rapidly to reach at 2h values higher than (myod1) or similar to (myog, pcna, fmhc, inos, nnos, ctsd) initial values (0h). Transcript levels then decreased slowly up to 5h (myog) or 7h (myod1) or did not change until 7h (pcna) and finely increased between 7h and 9h (myod1, myog, pcna) to reach values higher than (myod1, pcna) or similar to (myog) initial values (0h). The gene expression of fmhc, inos, nnos and ctsd did not change significantly between 2h and 9h in C-fry.

In N-fed fry (Figures 8,9), the transcript level of pcna did not decrease between 0h and 1h, and was lower at 0h, 3h, 9h than at 7h. Myod1 expression was upregulated 1h and 2h after distribution of the single meal, decreased at 3h, and then not significantly changed. Fmhc expression remained stable between 0h and 2h, decreased at 3h, increased at 5h and then did

not change. Inos expression decreased between 1h and 3h and then remained stable. Ctsd expression was higher at 1h than at 9h. Myog and nnos expressions did not vary significantly after the single meal.

Compared to C-fed fry, the N-fed fry had higher expressions of pcna at 1h, 7h, of myod1 at 1h, of fmhc at 0h, 1h, 2h, 5h, 9h, of inos at 0h, 1h, 5h, of nnos at 0h, 1h, 3h, 5h, 7h, of ctsd at 0h, 1h, 7h, and lower expressions of pcna at 0h, 9h and of Myog at 0h, 2h, 3h, 9h (Figures 8,9).

3.2.3. Recruitment and growth dynamics of skeletal white muscle fibres

In the dorsal muscle of 20 days fed fry, white muscle fibres presented a main stratified spatial organisation, with numerous small white fibres located at white muscle epi-axial extremity and at white muscle edge under red muscle, and biggest white fibres located in the deep part of myotomes. Small white muscle fibres were also present in the deep part of white muscle, around largest fibres (Figure 10). Twenty days of feeding with the N-diet led to a significant decrease in white muscle total cross-sectional area (30% lower than in C-fed fry) and total number of white muscle fibres (17% lower than C-fed fry) but did not change the mean diameter of white muscle fibres (Table 4). The comparison of the distributions of muscle fibre diameters in C- and N-fed fry showed that feeding the N-diet led to a lowering of muscle fibre number in almost all diameter classes (Figure 11). In the 5-7.5 µm and 10-12.5µm classes, this decrease failed to be significant ($P=0.097$ and $P=0.072$, respectively) due to high individual variability. The N-induced decrease in white fibre number was significant for the 12.5-15µm ($P<0.001$), 17.5-20µm ($P=0.047$), 20-22.5 µm ($P=0.030$) and 22.5-25µm ($P=0.010$) classes of diameters.

4. Discussion

Embryonic myogenesis and Arg or L-NAME baths

The rate of somitogenesis observed here (5.7 somites formed/day at 8 °C, i.e. 0.7 somite formed/degree-day) is consistent with previous observations in the same species (Bobe et al., 2000). Other data with rainbow trout have indicated that the initial differentiation of fast (white) muscle takes place in proximity to axial structures and that fmhc transcript is detected from 25 somites onwards (Rescan et al., 2001). Our results allowed to precise that fmhc expression began earlier, as soon as 96°dpf (12 dpf at 8 °C), i.e. in 19-somite embryo. Concerning myf5 transcript, we report here for the first time its presence in trout at 72 °dpf (9 dpf at 8 °C) at the level of paraxial presomitic mesoderm, i.e. before the beginning of the morphological differentiation of somites. This early expression of myf5 is consistent with its known function in myogenic determination (Buckingham and Rigby, 2014).

The free Arg content presently found in control eggs at 13dpf (0.52µM/g) is consistent with previous data on the same species (Rice and Stokes, 1974). Immersion during 4h in a bath containing 2mM/L Arg allowed to double the free Arg content of eggs, confirming that trout eggs might be enriched in some nutrients through delivery in embryonic baths, as yet shown with other water-soluble nutrients (Falahatkar et al., 2006). We enriched trout eggs with Arg in order to stimulate myogenic proliferation, act on the expression of genes regulating myogenic steps and increase the number of muscle fibres, based on results obtained in chicken hatched from eggs injected with Arg, in foetuses from gilts fed diets supplemented with Arg during early gestation, and in growing-finishing pigs from sows fed diets supplemented with Arg during early gestation (Bérard and Bee, 2010; Kalbe et al., 2013; Shi et al., 2018 ; Subramaniyan et al., 2019). In present conditions, in spite of increasing trout egg

488 free Arg content, an embryonic bath in 2mM/L Arg performed at 13dph (when 21 somites
489 were formed) failed to modify significantly the embryonic pattern of expression of myf5 and
490 fmhc, and the number of white muscle formed at hatching. An earlier bath (at 9dph, just
491 before the beginning of somitogenesis) in 2mM/L Arg also did not act on these myogenic
492 markers. Perhaps the concentration of 2mM/L Arg, chosen for its ability to stimulate *in vitro*
493 the activation of mice satellite cells (Betters et al., 2008), was not sufficient to induce changes
494 *in vivo* on the myogenic markers studied, or the duration of Arg treatment too short. We used
495 embryonic L-NAME bath in order to determine if treatment of trout eggs with a NOS
496 inhibitor would act on the fate of muscle precursor cells and on muscle cellularity, as it occurs
497 *in vivo* in mice and rats injected intraperitoneally with L-NAME or fed with L-NAME in
498 drinking water and in chick injected *in ovo* by NPLA (another NOS inhibitor) (Anderson et
499 al., 2000; Carrazo et al., 2014; Tatsumi et al., 2006; Wang et al., 2001). We chose to use a low
500 concentration of L-NAME (1mM/L) as we did not want to alter embryo survival and this aim
501 was attained, confirming previous results (Eddy et al., 1999), but this treatment did not alter
502 neither the embryonic pattern of expression of myf5 and fmhc nor the number of white
503 muscle fibres formed at hatching. Perhaps, like for 2mM/L Arg, 1mM/L L-NAME was too
504 low to alter the activation of muscle precursor cells, their differentiation, and the formation of
505 muscle fibres, or bath duration too short. Perhaps also, despite the described occurrence of
506 nNOS transcripts in the tail bud of zebrafish embryos (Yamamoto et al., 2003) and of NOS
507 positive cells in rainbow trout embryos (Gallo and Civinini, 2001), NOS activity was too low
508 in 9 and 13 dpf trout embryos to respond to an external stimulation by the tested doses of Arg
509 and L-NAME. This latter hypothesis led us to try to modify myogenesis by acting on NOS
510 activity later in development. Our second experiment was thus performed on first feeding fry,
511 as the weeks following first feeding constitute in trout a time window during which i) an
512 important recruitment of white muscle fibres occurs and ii) a nutritional control of

myogenesis has been demonstrated at molecular and cellular levels (Alami-Durante et al., 2014; Fontagné-Dicharry et al., 2017).

Pre- and post-prandial expressions of myogenic and muscle growth-related genes in fry fed from first feeding onwards and during 20 days a diet covering nutritional requirements

Although the temporal changes in expression of myogenic and muscle growth-related genes that occurred after a long starvation period followed by a period of refeeding of numerous days/weeks have been documented in different species of farmed fish (e.g. Bower et al., 2009; Chauvigné et al., 2003; Cleveland and Weber, 2014; Dhillon et al., 2009; Fuentes et al., 2012; Garcia de la serrana et al., 2012; Hagen et al., 2009; Johansen and Overturf, 2006; Paula et al., 2017; Rescan et al., 2007, 2017, Zhu et al., 2014), these data only concern juveniles and less data are available for shorter time intervals. To our knowledge, the effect of the distribution of a single meal on the kinetics of transcription of myogenic genes was only studied in juveniles of species of interest to aquaculture (Valente et al., 2012; Zhu et al., 2016) or in model fish (Seiliez et al., 2013) previously submitted to several days of fasting. Our study is the first one undertaken under farming conditions with just an overnight fast and dealing with fry. We found that the expressions of pcna (necessary for cell proliferation) and fmhc (a myosin chain expressed in white muscle) were downregulated 1h after refeeding, and the expressions of the myogenic factors myod1 (involved in activation of muscle precursor cells, MPC) and myog (involved in early differentiation and fusion of MPC), of nnos (the constitutive nos isoform providing low levels of nitric oxide under physiological conditions) and ctsd (involved in lysosomal proteolysis) followed, although not significantly, the same trend. The expression of the isoform of nos providing high level of nitric oxide under stress conditions (inos) was very similar before the single meal and 1h later, suggesting that the

down regulation of the expression of other genes at 1h post feeding was not due to stress. It was perhaps due to a transitory down regulation of the transcription of genes involved in myogenesis and muscle growth at the expense of transcription of genes involved in digestion and absorption of nutrients. The rapid upregulation of the transcription of myogenic regulatory factors and myosin heavy chain occurring between 1h and 2h after the single meal was for its part probably triggered by the delivery of absorbed nutrients into muscle cells, and the subsequent progressive downregulation of myogenic regulatory factor transcripts linked to a lowering of nutrient delivery when gut fullness decreased. The kinetics of post-prandial changes in myod1 and myog expressions here found in trout fry after a single meal are not similar to known kinetics in juveniles of other aquaculture fish species after a single meal (Valente et al., 2012; Zhu et al., 2016). This discrepancy might be related to factors intrinsic to the fish (species, developmental stage, size) and to the experimental design (starvation/refeeding protocol, diet composition, rearing temperature, post-prandial sampling times). The post-prandial changes in myod1 and myog expressions here observed are the result of nutrient input after just an overnight fasting, while those previously observed (Valente et al., 2012; Zhu et al., 2016) are based on the effects of feeding after one week of fasting, when fish are in a catabolic state.

Effect of L-NAME feeding on fry growth, pre- and post-prandial gene expressions, and muscle cellularity

The decreased weight of N-fed fry after 3 weeks of feeding with maintenance of a length-weight relation similar in slope and origin to that of C-fed fry indicates that L-NAME-feeding although led to reduced somatic growth of fry, did not modify their growth trajectory. Taking into account the quantity of feed delivered ad-libitum during the whole trial, the mean

563 weight of fry during the trial ((final fry weight + weight of dead fry - initial fry weight)/2) and
564 trial duration, the fry had ingested 100mg L-NAME/kg body weight/day, *i.e.* an L-NAME
565 quantity higher than that (30mg L-NAME/kg body weight/day) decreasing the formation of
566 new myotubes in mouse (Anderson, 2000) and satellite cell activation in rat (Tatsumi et al.,
567 2006), and close to that (90mg L-NAME/kg body weight/day) reducing myonuclear addition
568 in rat (Gordon et al., 2006). Long-term L-NAME feeding of trout fry increased the pre-
569 prandial gene expression level of nnos and inos. Inos expression also increased in different
570 mammalian cell types after chronic treatment with L-NAME (e.g. Kopincova et al., 2012;
571 Miller et al., 1996), and it was suggested that L-NAME induced a feedback-regulated
572 activation of nos expression which could, *in vivo*, be or not be associated with changes in NO
573 availability, depending on different factors such as duration of treatment, dose of L-NAME,
574 presence of NOS cofactors, and tissue (Kopincova et al., 2012). In trout fry, long-term L-
575 NAME feeding also increased the pre-prandial gene expression level of fmhc and ctsd, which
576 could be indicative of increased protein turnover, but it decreased the pre-prandial gene
577 expression level of pcna and myog, suggesting decreased cell proliferation and early
578 differentiation and fusion of myoblasts. These findings are in line with *in vitro* results
579 showing that L-NAME reduces the number of proliferating satellite cells on mice single fibre
580 cultures (Buono et al., 2012) and reduces the fusion of C_2C_{12} myoblasts (Long et al., 2006). In
581 trout fry, long-term L-NAME feeding also supressed the peak of expression of pcna, myog,
582 and fmhc occurring 2 hours after feeding in control fed fry. The L-NAME-induced molecular
583 changes led, at cellular phenotype level, to a lower total number of white muscle fibres and to
584 a lower number of large muscle fibres, indicating that L-NAME feeding had decreased fry
585 muscle growth by altering both fibre recruitment (hyperplasia) and fibre growth
586 (hypertrophy). This suggests, based on the recognised inhibitory role of L-NAME on NOS

activity, that NO synthesis, yet recognised as being important for the early myogenesis of mammals and birds, is also important for fish fry myogenesis.

5. Conclusion

We observed that manipulating the arginine-NO pathway during embryogenesis did not have any significant impact on early myogenic processes in rainbow trout. Feeding trout from first feeding onwards with a L-NAME enriched diet induced alterations in the kinetics of transcription of nos, myogenic and muscle-growth related genes. These observations combined with L-NAME-induced decreased muscle growth, fibre recruitment and fibre hypertrophy support the hypothesis of an involvement of NO in the early myogenesis of fish.

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Author contributions

H.A-D. and S.K. designed the research. M.C. and D.B. realized the molecular and cellular analyses of muscle. C.V. helped during sampling and quantified the free Arg content of eggs. H.A-D. analysed the data, wrote the paper and has primary responsibility for final

611 content. We sincerely regret the demise of one of the co-authors (M.C.). Other authors have
612 read and approved the final manuscript.
613
614 **Declaration of Competing Interest**
615
616 The authors have no competing interests to declare.
617

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Figure captions

Figure 1.

Schematic diagram of experiments 1 and 2 with treatments and sampling stages.

Figure 2.

Somitogenesis in trout embryos.

Figure 3.

Expression of myf5 in trout embryos treated at 9 dpf or 13 dpf by immersion in C, A or N-bath. Whole mount *in situ* hybridization of embryos at 9, 10, 12, 14, 18 and 22 dpf.

Figure 4.

Expression of fmhc in trout embryos treated at 9 dpf or 13 dpf by immersion in C, A or N-bath. Whole mount *in situ* hybridization of embryos at 12, 14, 18 and 22 dpf.

Figure 5.

Growth of the fry hatched from embryos treated at 9 dpf or 13 dpf by immersion in C, A or N-bath. Values are mean ± SD (n=3).

Figure 6.

Total length and body weight of the C- and N-fed fry after 20 days of feeding.

Values are mean ± SEM (n=63).

Figure 7.

960 Length-weight relationships of the C- and N-fed fry after 20 days of feeding (n= 63).

961 TL, total length; W, body weight.

962

963 Figure 8.

964 Pre- and post-prandial expressions of myogenic regulatory factors (myod1, myog),
965 proliferative cell nuclear antigen (pcna) and fast myosin heavy chain (fmhc) in C- and N-fed
966 fry after distribution of a single meal.

967 Values are mean ± SEM (n=9) of NORMA-gene normalized transcript levels. Within C-
968 samples, means with unlike lower-case letter are significantly different ($P<0.05$). Within N-
969 samples, means with unlike upper-case letter are significantly different ($P<0.05$). Within time
970 points, symbols indicate diet-induced statistical differences (***$P<0.001$; **$P<0.01$;
971 *$P<0.05$).

972

973 Figure 9.

974 Pre- and post-prandial expressions of inducible nitric oxide synthase (inos), neuronal nitric
975 oxide synthase (nnos) and cathepsin D (ctsd) in C- and N-fed fry after distribution of a single
976 meal.

977 Values are mean ± SEM (n=9) of NORMA-gene normalized transcript levels. Within C-
978 samples, means with unlike lower-case letter are significantly different ($P<0.05$). Within N-
979 samples, means with unlike upper-case letter are significantly different ($P<0.05$). Within time
980 points, diet-induced statistical differences are indicated by symbols (***$P<0.001$; **$P<0.01$;
981 *$P<0.05$).

982

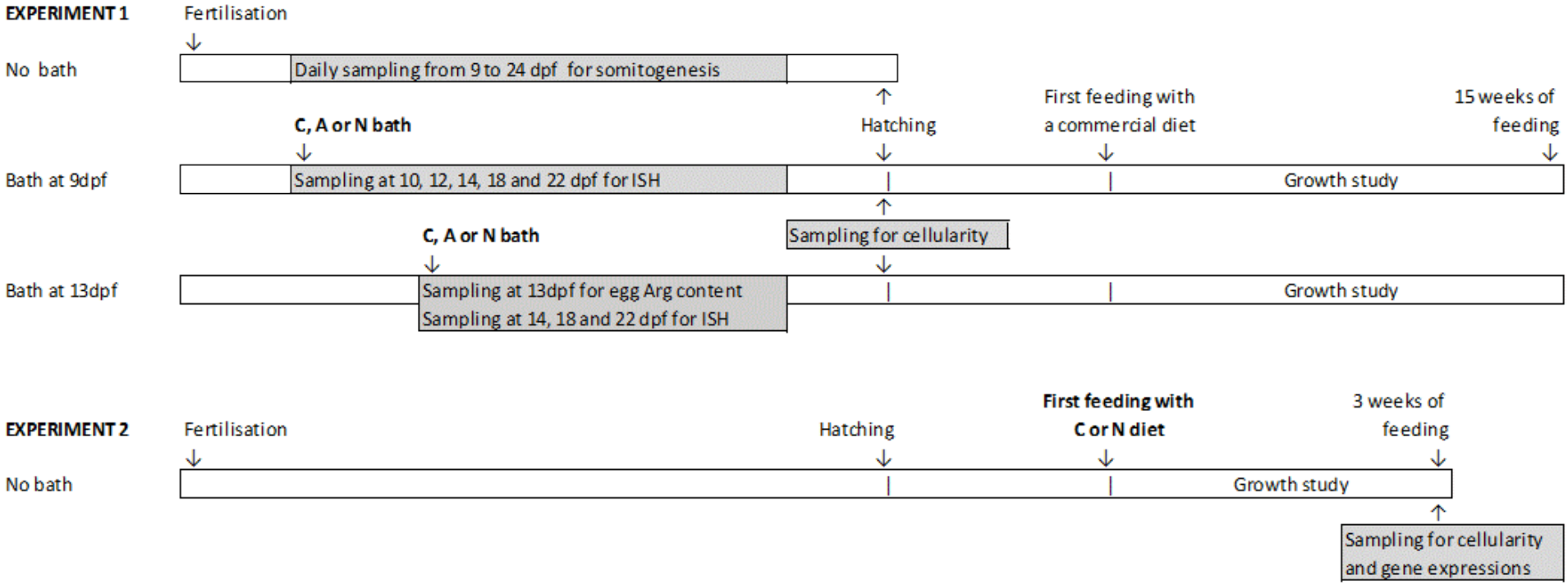
983 Figure 10.

Transverse dorsal section of a trout fry fed for 20 days with the C-diet showing the position of white muscle fibres with an area lower than 100µm² (in red), between 100 and 240 µm² (in blue) and larger than 240µm² (in green).

WM white muscle, *RM* red muscle, *m* myosept, *SC* spinal cord, *NC* notochord, *hs* horizontal septum, *s* skin. Scale bar = 100 µm.

Figure 11.

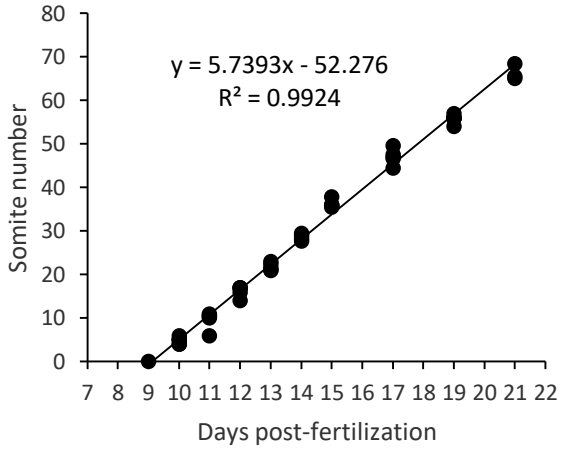
Distribution of white skeletal muscle fibre diameters in the C- and N-fed fry after 20 days of feeding. Values are mean ± SEM (n=6). Within each diameter class, means not sharing a common letter are significantly different ($P<0.05$).



996

997 Figure 1.

998



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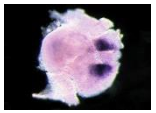
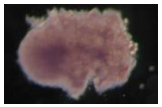
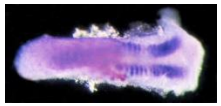
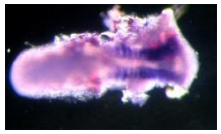
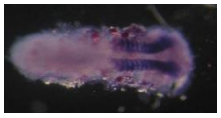
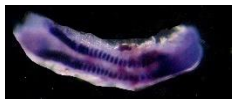
1000 Figure 2.

9dpf, before bath	Beginning of expression 		Sens probe (control) 			
9 dpf	C-bath	A-bath	N-bath	no bath	no bath	no bath
10 dpf						
12 dpf						
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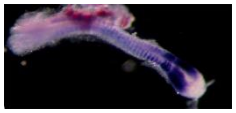
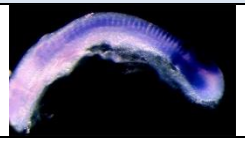
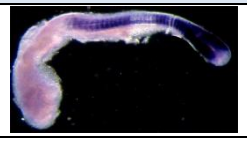
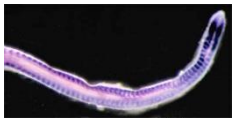
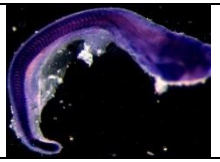
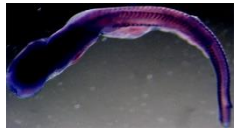
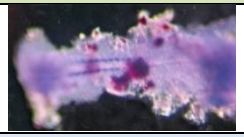
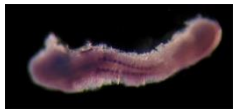
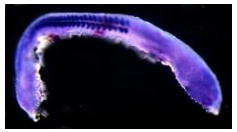
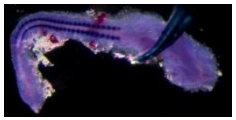
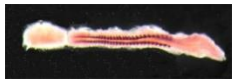
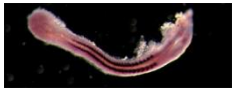
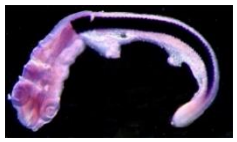
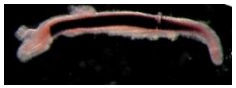
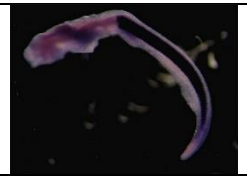
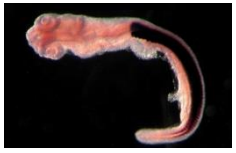
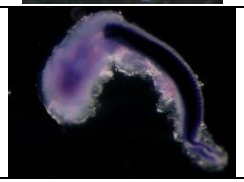
14 dpf						
18 dpf						
22 dpf						

Figure 3.

9 dpf	C-bath	A-bath	N-bath	no bath	no bath	no bath
12 dpf				Sens probe (control)		
13 dpf	no bath	no bath	no bath	C-bath	A-bath	N-bath
14 dpf						
18 dpf						
22 dpf						

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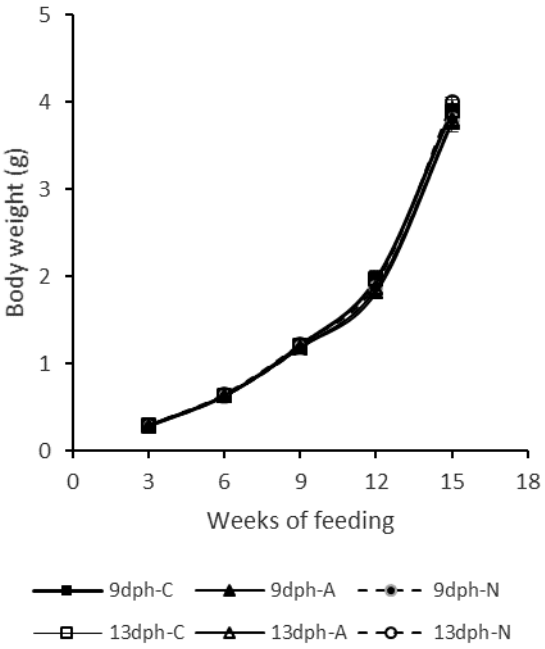

5
4
3
2
1
0
Body weight (g)
0
3
6
9
12
15
18
Weeks of feeding
9dph-C
9dph-A
9dph-N
13dph-C
13dph-A
13dph-N

Figure 5.

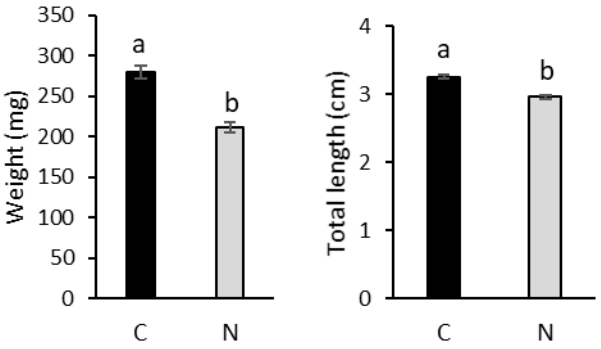

Weight (mg)
350
300
250
200
150
100
50
0
a
b
C
N
Total length (cm)
4
3
2
1
0
a
b
C
N

Figure 6.

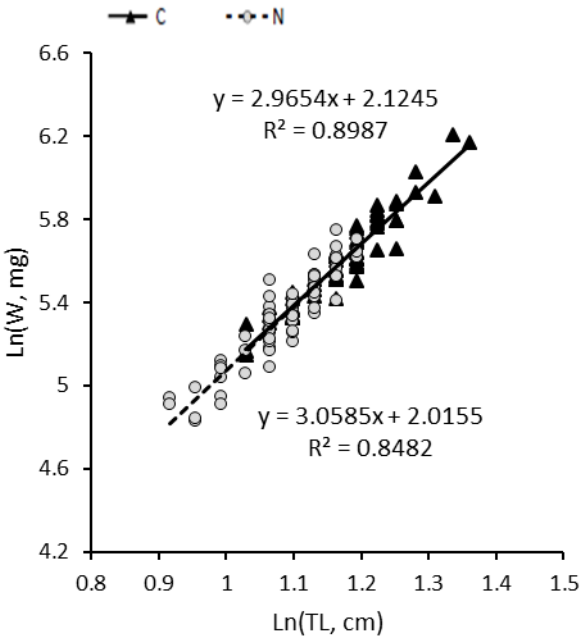

C
N
y = 2.9654x + 2.1245
R² = 0.8987
y = 3.0585x + 2.0155
R² = 0.8482
Ln(W, mg)
Ln(TL, cm)
6.6
6.2
5.8
5.4
5
4.6
4.2
0.8
0.9
1
1.1
1.2
1.3
1.4
1.5

Figure 7.

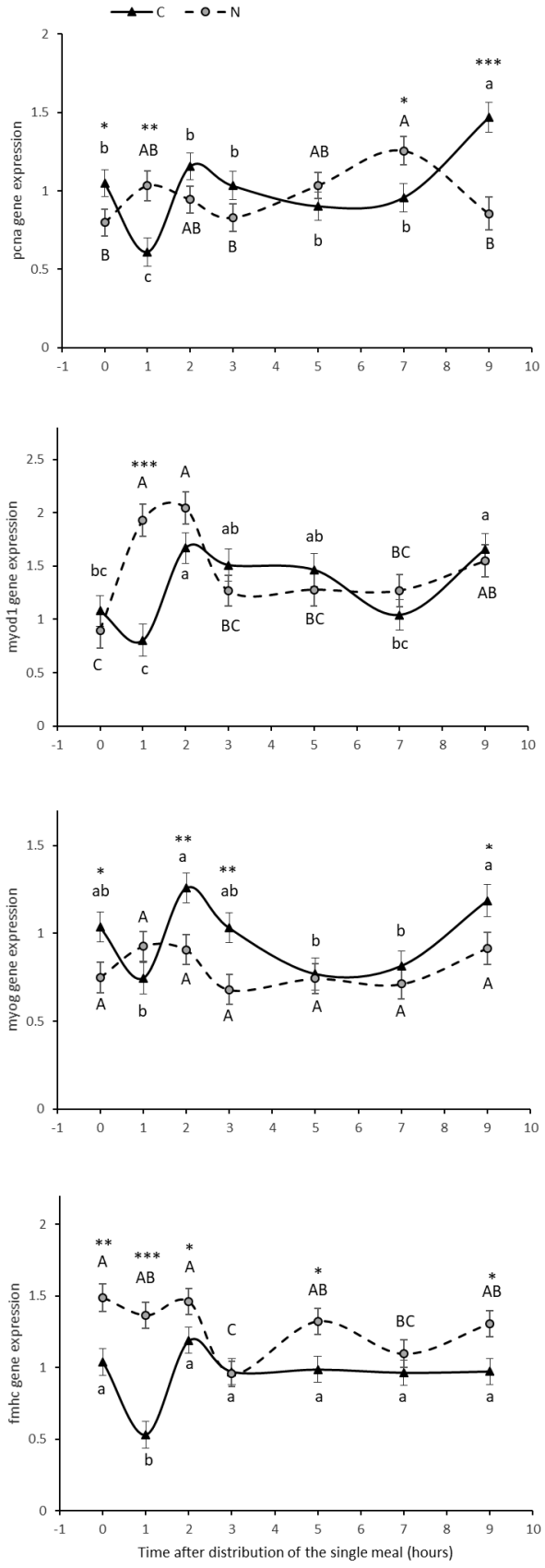

C
N
pcna gene expression
myod1 gene expression
myog gene expression
fmhc gene expression
Time after distribution of the single meal (hours)

Figure 8.

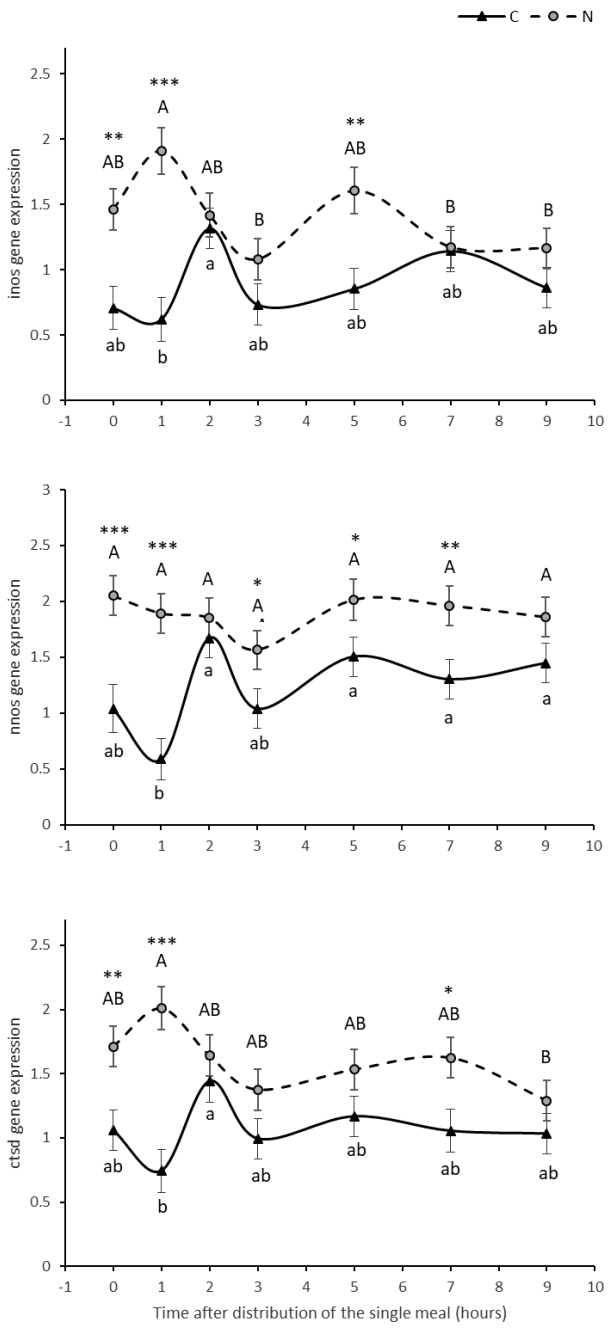

C
N
inos gene expression
nnos gene expression
ctsd gene expression
Time after distribution of the single meal (hours)

Figure 9.

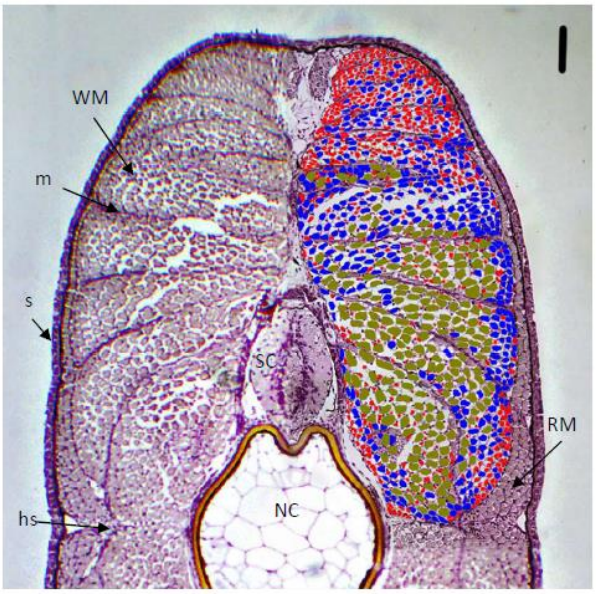


Figure 10.

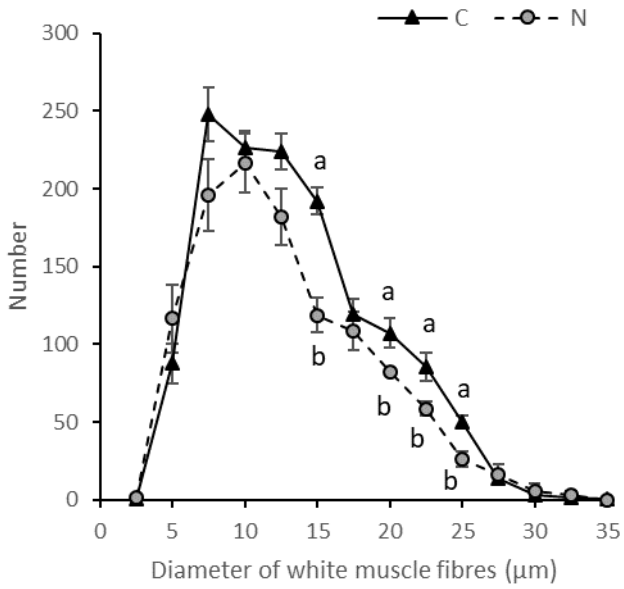

C
N
300
250
200
150
100
50
0
Number
0
5
10
15
20
25
30
35
Diameter of white muscle fibres (µm)
a
a
a
a
b
b
b
b

Figure 11.

Table 1.

Ingredient composition of the control (C) and L-NAME (N) diets.

Diets	C	N
Ingredients (g/100g diet)		
Fish soluble protein concentrate[1]	4.0	4.0
Wheat gluten[2]	5.0	5.0
Corn gluten meal[3]	38.0	38.0
Whole wheat[4]	29.6	29.6
Soy protein concentrate[5]	3.0	3.0
L-arginine	0.5	0.5
L-lysine	1.0	1.0
L-histidine	0.1	0.1
L-glutamate	0.8	0.8
Attractant mix[6]	1.5	1.5
$CaHPO_4.2H_2O$ (18%P)	3.5	3.5
Soy lecithin[7]	1.0	1.0
Fish oil[8]	9.0	9.0
Mineral premix[9]	1.5	1.5
Vitamin premix[10]	1.5	1.5
L-NAME[11]	0	0.1

[1] CPSP-90, Sopropèche (Wimille, France).

[2] Roquette (Lestrem, France).

[3] Inzo (Argentan, France)

[4] Sud-Ouest Aliment (Pomarez, France).

[5] Estril 75, Sopropèche (Wimille, France).

[6] Attractant mix (g/100g feed): Glucosamine 0.5g; taurine 0.3g, betaine 0.3g, glycine 0.2g, alanine 0.2g.

[7] Louis François (Croissy-Beaubourg, France).

[8] Sopropèche (Wimille, France).

[9] Mineral premix (g or mg/kg diet): calcium carbonate (40 % Ca), 2·15 g; magnesium oxide (60 % Mg), 1·24 g; ferric citrate, 0·2 g; potassium iodide (75 % I), 0·4 mg; zinc sulphate (36 % Zn), 0·4 g; copper sulphate (25 % Cu), 0·3 g; manganese sulphate (33 % Mn), 0·3 g; dibasic calcium phosphate (20 % Ca, 18 % P), 5 g; cobalt sulphate, 2 mg; sodium selenite (30 % Se), 3 mg; KCl, 0·9 g; NaCl, 0·4 g (UPAE, INRA, Jouy en Josas, France).

[10] Vitamin premix (μg or mg/kg diet): dl-α-tocopherol acetate, 60 mg; sodium menadione bisulphate, 5 mg; retinyl acetate, 4·5 mg; dl-cholecalciferol, 375 μg; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B_{12}, 0·05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2·5 mg; calcium pantothenate, 50 mg; choline chloride, 2000 mg (UPAE, INRA, Jouy en Josas, France).

[11] N5751, Sigma (France).

Table 2.

Analysed composition of the control diet (C).

Diet	C
Dry matter (DM, %)	90.6
Crude protein (% DM)	42.1
Total lipid (% DM)	8.3
Gross energy (kJ/g DM)	21.2
Essential amino acids (g/100g dry feed)	
Arginine	1.71
Histidine	0.76
Isoleucine	1.34
Leucine	4.32
Lysine	1.64
Methionine	0.75
Phenylalanine	1.95
Threonine	1.17
Tryptophan	0.22
Valine	1.54
Non-essential amino acids (g/100g dry feed)	
Alanine	2.39
Aspartic acid	2.14
Cysteine	0.58
Glutamic acid	8.37
Glycine	1.38
Proline	2.98

Serine	1.76
Tyrosine	1.34

Table 3

Survival, body weight and muscle cellularity at hatching of the fry hatched from embryos immerged at 9 or 13dpf in C, A or N bath.

	Treatments[1]						*P-value*		
	9dpf-C	9dpf-A	9dpf-N	13dpf-C	13dpf-A	13dpf-N	dpf	bath	dpf*bath
Survival[2]	94.6±0.6	93.2±1.1	94.2±1.5	94.1±1.1	93.9±0.9	94.9±0.2	0.411	0.184	0.569
BW[3]	46.3±1.7	48.8±2.1	43.0±1.0	43.6±1.3	46.3±1.8	47.2±1.2	0.917	0.257	0.053
TCSAWM[4]	52495±4588	59797±4588	71429±4588	63586±5026	56914±4248	59833±4588	0.742	0.331	0.077
TNWF[5]	768±65	840±65	787±65	923±71	836±60	808±54	0.272	0.768	0.419

[1]9dpf-C immersion in C-bath at 9dpf; 9dpf-A immersion in A-bath at 9dpf; 9dpf-N immersion in N-bath at 9dpf; 13dpf-C immersion in C-bath at 13dpf; 13dpf-A immersion in A-bath at 13dpf; 13dpf-N immersion in N-bath at 13dpf.

[2]Survival (%), values are means ± SD (n=3 tanks). [3]BW, body weight (mg), values are means ± SE (n=27 fry). [4]TCSAWM, total cross-sectional area of white muscle (µm²), values are means ± SE (n=6 fry). [5]TNWF, total number of white muscle fibres, values are means ± SE (n=6 fry).

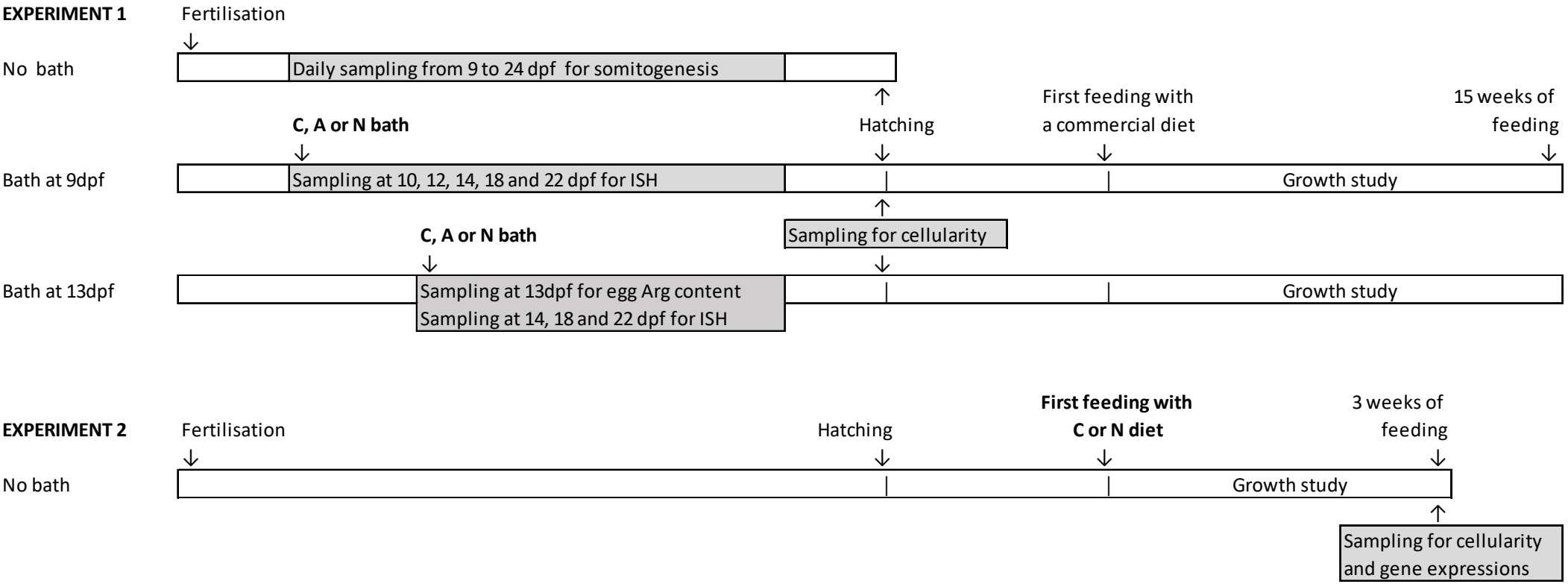
Table 4.

Cellularity of the dorsal skeletal white muscle of fry fed with the C- or N-diet for 20 days.

	Diet		
	C	N	*P*-value
TCSAWM (µm²)	419959 ± 21455 a	293028 ± 9651 b	<0.001
TNWF	1553 ± 56 a	1283 ± 135 b	0.009
d mean (µm)	12.2 ± 0.2	11.7 ± 0.5	0.383

TCSAWM, total cross sectional area of white muscle; TNWF, total number of white muscle fibres; d, diameter of white muscle fibres.

Values are means ± SEM (n=6). Within each row, means not sharing a common superscript letter are significantly different.


EXPERIMENT 1
Fertilisation
No bath
Daily sampling from 9 to 24 dpf for somitogenesis
Hatching
First feeding with a commercial diet
15 weeks of feeding
C, A or N bath
Bath at 9dpf
Sampling at 10, 12, 14, 18 and 22 dpf for ISH
Growth study
Sampling for cellularity
C, A or N bath
Bath at 13dpf
Sampling at 13dpf for egg Arg content
Sampling at 14, 18 and 22 dpf for ISH
Growth study
EXPERIMENT 2
Fertilisation
Hatching
First feeding with C or N diet
3 weeks of feeding
No bath
Growth study
Sampling for cellularity and gene expressions