

## RESEARCH ARTICLE

# Ontogenetic expression of metabolic genes and microRNAs in rainbow trout alevins during the transition from the endogenous to the exogenous feeding period

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### SUMMARY

As oviparous fish, rainbow trout change their nutritional strategy during ontogenesis. This change is divided into the exclusive utilization of yolk-sac reserves (endogenous feeding), the concurrent utilization of yolk reserves and exogenous feeds (mixed feeding) and the complete dependence on external feeds (exogenous feeding). The change in food source is accompanied by well-characterized morphological changes, including the development of adipose tissue as an energy storage site, and continuous muscle development to improve foraging. The aim of this study was to investigate underlying molecular mechanisms that contribute to these ontogenetic changes between the nutritional phenotypes in rainbow trout alevins. We therefore analyzed the expression of marker genes of metabolic pathways and microRNAs (miRNAs) important in the differentiation and/or maintenance of metabolic tissues. In exogenously feeding alevins, the last enzyme involved in glucose production (*g6pca* and *g6pcb*) and lipolytic gene expression (*cpt1a* and *cpt1b*) decreased, while that of *gk*, involved in hepatic glucose use, was induced. This pattern is consistent with a progressive switch from the utilization of stored (gluconeogenic) amino acids and lipids in endogenously feeding alevins to a utilization of exogenous feeds via the glycolytic pathway. A shift towards the utilization of external feeds is further evidenced by the increased expression of *omy-miRNA-143*, a homologue of the mammalian marker of adipogenesis. The expression of its predicted target gene *abhd5*, a factor in triglyceride hydrolysis, decreased concurrently, suggesting a potential mechanism in the onset of lipid deposition. Muscle-specific *omy-miRNA-1/133* and *myod1* expression decreased in exogenously feeding alevins, a molecular signature consistent with muscle hypertrophy, which may be linked to nutritional cues or increased foraging.

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### INTRODUCTION

Recent molecular tools have allowed for a more detailed understanding of the nutritional and endocrine regulation of the intermediary metabolism in adult rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)]. Several studies of key metabolic tissues of adult rainbow trout have elucidated molecular mechanisms involved in metabolic regulation in the liver (Plagnes-Juan et al., 2008; Mennigen et al., 2012), muscle (Seiliez et al., 2011; Gabillard et al., 2010), adipose tissue (Polakof et al., 2011; Cruz-Garcia et al., 2012), brain (Polakof et al., 2007; Polakof et al., 2008) and Brockmann bodies (Polakof et al., 2008). These experiments largely support the notion from mammalian model systems that, albeit not exclusively, many metabolic pathways are finely regulated at the level of gene expression of key enzymes (Desvergne et al., 2006). In addition to these 'classical' metabolic genes, recent studies in mammalian model systems have shown that microRNAs (miRNAs) constitute important regulators of intermediary metabolism in several tissues (Rottiers and Näär, 2012). This finding is not surprising, as miRNAs have been shown to postranscriptionally regulate metabolic target genes directly or indirectly through important signaling pathways involved in the coordination of cellular metabolism (Tibiche and Wang, 2008). Indeed, preliminary evidence suggests that their roles in fish metabolism may be conserved (Her et al., 2011; Mennigen et al., 2012), in line with the

highly conserved nature of many miRNAs in the vertebrate lineage (Lee et al., 2007). With this increasing availability of molecular tools, we wanted to determine for the first time the underlying molecular mechanisms (mRNAs and miRNAs) that are associated with the transition between endogenous (vitellus) and exogenous (diet) feeding in rainbow trout alevins. The understanding of nutrition in fish larvae and/or alevins is not only an important question in developmental biology, but also has critical importance to aquaculture, as the lack of its understanding is considered to be one of the contributing factors in the high larval mortality observed in commercial fish rearing (Li and Leatherland, 2008; Conceição et al., 2010).

The present study therefore specifically aimed to investigate expression changes of genes and miRNAs associated with intermediary metabolism in rainbow trout alevins, which undergo a rapid ontogenetic switch in nutritional strategies (Vernier, 1969) that is characteristic of oviparous fish development (Balon, 1986). Briefly, the succession of nutritional strategies in rainbow trout progresses from an exclusive reliance on endogenous yolk-sac reserves (Vernier stage 1–35) to a stage of mixed feeding after the first meal (Vernier stage 36), which is characterized by the concurrent use of exogenous food and remaining yolk-sac reserves, to an exclusively exogenous nutrition (Vernier stage >37). However, little is known regarding the potential molecular regulation of metabolic pathways in this process,

or the potential role of miRNA in the acquisition of the capacity to utilize external feeds. Specifically, we assessed potential changes in global intermediary metabolism between nutritional phenotypes by investigating the expression of genes of glucose metabolism (glycolysis and gluconeogenesis), lipid metabolism (lipogenesis and  $\beta$ -oxidation) and amino acid metabolism (amino acid catabolism). Additionally, we investigated the expression of specific miRNAs and their predicted target genes, whose function in metabolic tissues has been characterized in mammalian models (Rottiers and Näär, 2012). The miRNAs investigated in this study have either been associated with the differentiation and function of specific metabolic tissues in mammals, such as a role for miR-33 and miR-122 in proliferation and lipid metabolism in the liver (Cirera-Salinas et al., 2012; Elmén et al., 2008), a role for miRNA-143 in the differentiation of adipose tissue (Esau et al., 2004) and miR-1/133 in the differentiation of muscle (Chen et al., 2006), or are ubiquitously expressed in mammalian metabolic tissues, where they have important metabolic roles in the regulation of insulin signaling, such as miR-29 (Pandey et al., 2011; Rottiers and Näär, 2012) and miR-103/107 (Trajkovski et al., 2011). Indeed, the recent identification of several conserved rainbow trout homologues of these miRNAs (Salem et al., 2010) and the postprandial hepatic regulation of some of these miRNAs in rainbow trout suggest a conserved metabolic role for these miRNAs in rainbow trout (Mennigen et al., 2012). Furthermore, components of the miRNA pathway have been shown to be expressed in early trout development, and the distinct regulation of some miRNAs in early trout ontogenesis indicates their functional involvement in trout developmental processes (Ramachandra et al., 2008).

## MATERIALS AND METHODS

### Fish and experimental design

The experiments were carried out in accordance within the clear boundaries of EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e. Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decret no. 2001-464, 29 May 2001). Rainbow trout embryos were initially reared from 16 November 2011 at INRA experimental facilities at Les-Athas, France, in 8°C stream water up to the age of 64 days [ $8^{\circ}\text{C} \times 64 \text{ days} = 512 \text{ degree days } (^{\circ}\text{D})$ ], during which time they hatched at the age of 44 days ( $=352^{\circ}\text{D}$ ). Following this, the alevins were transferred to the experimental facilities at INRA Donzac, France, and distributed into a 501 tank of oxygenated spring water maintained at 18°C. Unfed fish with visible yolk sacs were sampled at the age of 65 days ( $8^{\circ}\text{C} \times 64 \text{ days} + 18^{\circ}\text{C} \times 1 \text{ day} = 550^{\circ}\text{D}$ , Vernier stage 35);  $N=9$  independent samples were collected, of which each sample consisted of five pooled fish (Fig. 1). Both groups (embryos and alevins) were subsequently used for gene expression analysis of mRNA and miRNA ( $N=9$ ), respectively. Following the emergence of the first free-swimming fish, the second group of trout that still exhibited small yolk reserves (71 days,  $8^{\circ}\text{C} \times 64 \text{ days} \times 18^{\circ}\text{C} \times 7 \text{ days} = 638^{\circ}\text{D}$ , Vernier stage 36) was fed a first meal at 09:00h and killed 3h after the meal (corresponding well to the postprandial period in alevins) by terminal anesthetization by bathing in benzocaine prior to pooling and storage in liquid nitrogen. The fed meal consisted of a commercial diet of 0.5 mm diameter pellets (Skretting, Fontaine-les-Vervins, France; fishmeal 51.6%, fish oil 13.1%, corn starch 13.1%, wheat gluten 10.5%, pea protein concentrate and 10.5% mineral mix). Finally, 84-day-old ( $892^{\circ}\text{D}$ ) alevins, which were characterized by a complete resorption of the yolk sac (Vernier stage  $>37$ ) and had been maintained on a continuous feeding regime of multiple meals a day, were killed 3h following feeding as described above. Care was taken to maintain the same

daily time frame before sampling to avoid potential circadian effects, known to affect metabolism in rainbow trout (Bolliet et al., 2000). Therefore, fish were consistently sampled at 12:00h for each ontogenetic stage investigated in this study. Additionally, the time frame between feeding and sampling was deliberately chosen based on previous experiments showing potent postprandial activation of metabolic gene expression and miRNA expression 2–4h after a meal in the liver of rainbow trout (Mennigen et al., 2012). The samples were stored at  $-80^{\circ}\text{C}$  until utilized for analysis.

### Relative quantification of mRNA and omy-miRNA

Relative whole alevin gene expression of mRNA and miRNA was determined by quantitative real-time RT-PCR. The extraction of total RNA from whole alevins was performed using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis. The NCode VILO miRNA cDNA Synthesis Kit (Invitrogen) and the SuperScript III RNaseH-Reverse Transcriptase Kit (Invitrogen) with random primers (Promega, Charbonnières, France) were used according to the manufacturer's protocol to synthesize cDNA ( $N=9$  for each time point) for miRNA and mRNA, respectively. For gene expression assays, forward primer sequences for miRNAs were taken directly from the sequence information provided by Salem and colleagues (Salem et al., 2010), or, if not available, designed based on miRNA sequences found in miRBase (www.mirbase.org). The reverse primer used for all miRNA expression analysis was provided by Invitrogen with the NCode VILO miRNA cDNA Synthesis Kit. The primer sequences used in the real-time RT-PCR assays for mRNA of metabolic genes, as well as the protocol conditions of the assays, have been previously published (Alami-Durante et al., 2010; Kolditz et al., 2010; Prindiville et al., 2011; Seiliez et al., 2001). Primers for the  $\alpha/\beta$  hydrolase-domain 5 (*abhd5*) gene were newly designed using Primer3 (<http://frodo.wi.mit.edu/>) using the available trout sequence on the Gene Index Project (<http://compbio.dfci.harvard.edu/tgi/>) under accession number TC114063. Primer sequences for *abhd5* were CCAGAGGACTTCAACCACAGA (FW) and CCTCACAGATCACATGCTCAC (RV). To confirm specificity of the newly developed RT-PCR assay for *abhd5*, the amplicon was purified and sequenced (Beckman-Coulter Genomics, Takeley, UK). The specific forward primers used for miRNA real-time RT-PCR assays, as well as the specific temperatures used, are listed in Table 1. A universal poly T primer was used in all miRNA real-time RT-PCR assays as described in the manufacturer's protocol (NCode VILO miRNA cDNA Synthesis Kit, Invitrogen). For real-time RT-PCR assays of transcripts of both metabolic genes and miRNAs, the Roche Lightcycler 480 system was used (Roche Diagnostics, Neuilly-sur-Seine, France). The assays were performed using a reaction mix of 6  $\mu\text{l}$  per sample, each of which contained 2  $\mu\text{l}$  of diluted cDNA template, 0.12  $\mu\text{l}$  of each primer ( $10 \mu\text{mol l}^{-1}$ ), 3  $\mu\text{l}$  Light Cycler 480 SYBR Green I Master mix and 0.76  $\mu\text{l}$  DNase-/RNase-free water (5 Prime, Hamburg, Germany). The PCR protocol was initiated at  $95^{\circ}\text{C}$  for 10 min for initial denaturation of the cDNA and hot-start *Taq* polymerase activation, followed by 45 cycles of a two-step amplification programme (15 s at  $95^{\circ}\text{C}$ , 40 s at  $60\text{--}64^{\circ}\text{C}$ ), according to the primer set used. Melting curves were systematically monitored (temperature gradient at  $1.1^{\circ}\text{C } 10 \text{ s}^{-1}$  from  $65$  to  $94^{\circ}\text{C}$ ) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included replicate samples (duplicate of reverse

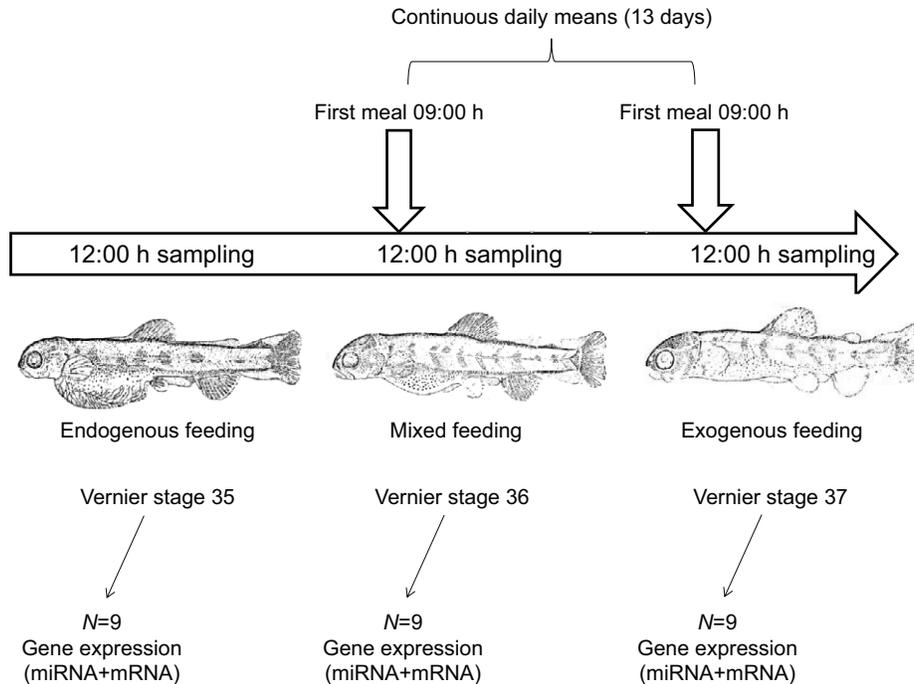


Fig. 1. Schematic representation of the experimental design. Trout alevins were sampled at three time points according to nutritional stages classified by drawings from Vernier (Vernier, 1969). Sampling was conducted at the same time (12:00 h) to avoid potential circadian effects. The last two samplings were conducted 3 h after trout were fed a commercial meal.

transcription and PCR amplification) and negative controls (reverse transcriptase- and cDNA template-free samples). The gene expression assays for the metabolic genes have been described previously in the publications previously cited for primer sequences. Briefly, the PCR protocol was initiated at 95°C for 3 min for initial denaturation of the cDNA and hot-start DNA polymerase activation and continued with 35 cycles of a two-step amplification programme (20 s at 95°C, 20 s at 56–60°C), according to the primer set used. Melting curves were systematically monitored (temperature gradient at 0.5°C 10 s<sup>-1</sup> 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 and controls as described above. For the expression analysis of both miRNA and mRNA, relative quantification of target gene expression was performed using the  $\Delta\Delta C_T$  method (Pfaffl, 2001). The relative gene expression of *18S* was used for the normalization of measured mRNAs and miRNAs, as its relative expression did not significantly change over sampling time (data not shown). In all cases, PCR efficiency ( $E$ ) was measured by the slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency values ranged between 1.8 and 2.2.

Table 1. Primer sequences used for real-time RT-PCR assays used to measure microRNA expression in whole rainbow trout alevins

omy-miRNA target	Forward primer sequence (5'–3')	Temperature (°C)
<i>omy-miRNA-1</i>	TGGAATGTAAAGAAGTATGTAT	61
<i>omy-miRNA-29</i>	GCACCATTTGAAATCCAGTGT	62
<i>omy-miRNA-33</i>	GTCATTGTAGTTGCATTGA	61
<i>omy-miRNA-103</i>	AGCATTGTACAGGGCTATCA	60
<i>omy-miRNA-107</i>	AGCATTGTACAGGGCTATGA	64
<i>omy-miRNA-122a</i>	TGGAGTGTGACAATGGTGTTTT	60
<i>omy-miRNA-122b</i>	TGGAGTGTGACAATGGTGTCT	60
<i>omy-miRNA-133</i>	TTGGTCCCTTCAACCAAGCTG	61
<i>omy-miRNA-143</i>	TGAGATGAAGCACTGTAGCTC	61

#### *In silico* prediction of miRNA-143 binding sites on trout *abhd5*

The *omy-miRNA-143* sequence was taken from Salem and colleagues (Salem et al., 2010) and rainbow trout sequence for *abhd5*, identified as a candidate target gene for *omy-miR-143* by Salem and colleagues, was retrieved from the trout expressed sequence tag database available on the Gene Index Project under accession number TC114063. To assess the potential for miRNA and mRNA interaction in more detail, several criteria were considered. These are specifically a thermodynamic stability of the RNA duplex assessed by median free energy, generally set at a minimum free energy (mFE) > -18 (Watanabe et al., 2006), the seed-rule stating that the 5' bases at position 2–7 of the miRNA are most important in mediating miRNA binding specificity and tolerate only a single mismatch (Brennecke et al., 2005), a preferential location in the 3' untranslated region (UTR) (Baek et al., 2008), and multiplicity of binding sites (Hon and Zhang, 2007). These parameters were assessed initially using RNAhybrid prediction software (Rehmsmeier et al., 2004) (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>) with a cut-off value of mFE < -18. In a second step, all sequences with more than one mismatch in the seed region were excluded. Lastly, the sequences were mapped on predicted coding and 3' UTR of the mRNA to assess the preferential regulation based on the location of the target site in the 3' UTR of the mRNA.

#### Statistical analysis

Data were analyzed by univariate one-way ANOVA. In cases where data were nonparametric or not homoscedastic, data transformations were used to meet ANOVA criteria. Normality was assessed using the Shapiro–Wilk test, while homoscedasticity was determined using Levene's test. Following univariate ANOVA analysis, the Student–Newman–Keuls test was used for *post hoc* analysis. Correlations between the expression of specific miRNAs and their respective target genes were analysed by Spearman's correlation test. Data were analyzed using SPSS version 17.0 (IBM, Armonk, NY, USA).

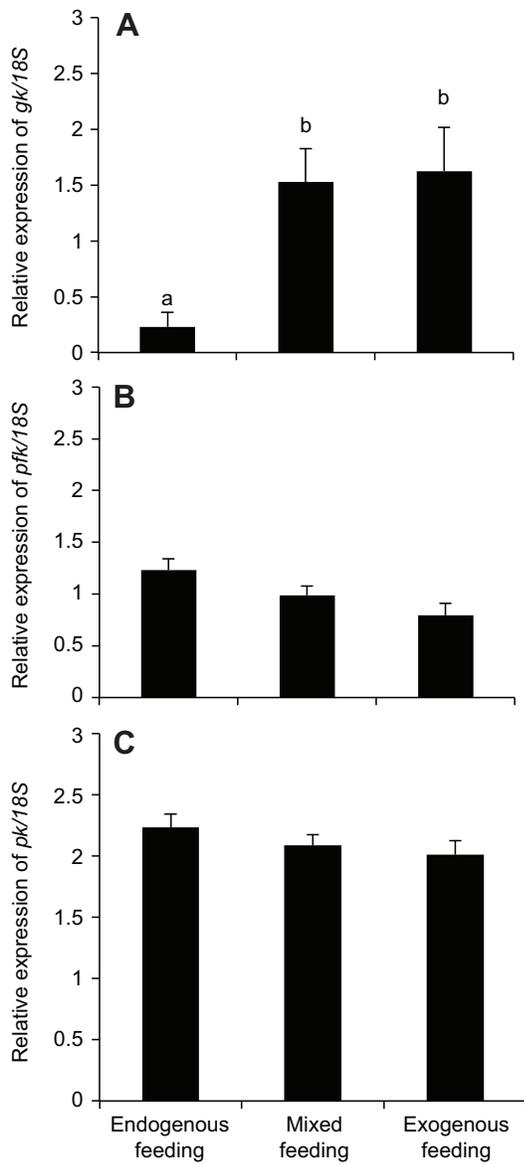


Fig. 2. Whole-body gene expression of genes involved in the intermediary metabolic pathway of glycolysis in alevins utilizing different nutritional strategies. Mean  $\pm$  s.e.m. gene expression data ( $N=9$ ) for (A) glucokinase, (B) phosphofructokinase and (C) pyruvatekinase are shown. Different letters indicate significantly different groups as established by one-way ANOVA and Student–Newman–Keuls *post hoc* test.

## RESULTS

### Decreased expression of glucose-6-phosphatase and $\beta$ -oxidation genes in trout alevins switching from endogenous to exogenous feeding

The expression of glucose metabolism-related genes revealed contrasting effects for some genes involved in the glycolytic (Fig. 2) and gluconeogenic pathways (Fig. 3). For glucokinase, the first enzyme involved in glucose use through phosphorylation, *gk* (d.f.=2,  $F=23.67$ ,  $P>0.01$ ), an increase in expression was observed at the mixed feeding and external feeding stages (Fig. 2A), while no difference in the gene expression of the two glycolytic enzymes, i.e. phosphofructokinase, *pfk* (d.f.=2,  $F=3.015$ ,  $P>0.05$ ), and pyruvate kinase, *pk* (d.f.=2,  $F=2.942$ ,  $P>0.05$ ), was observed

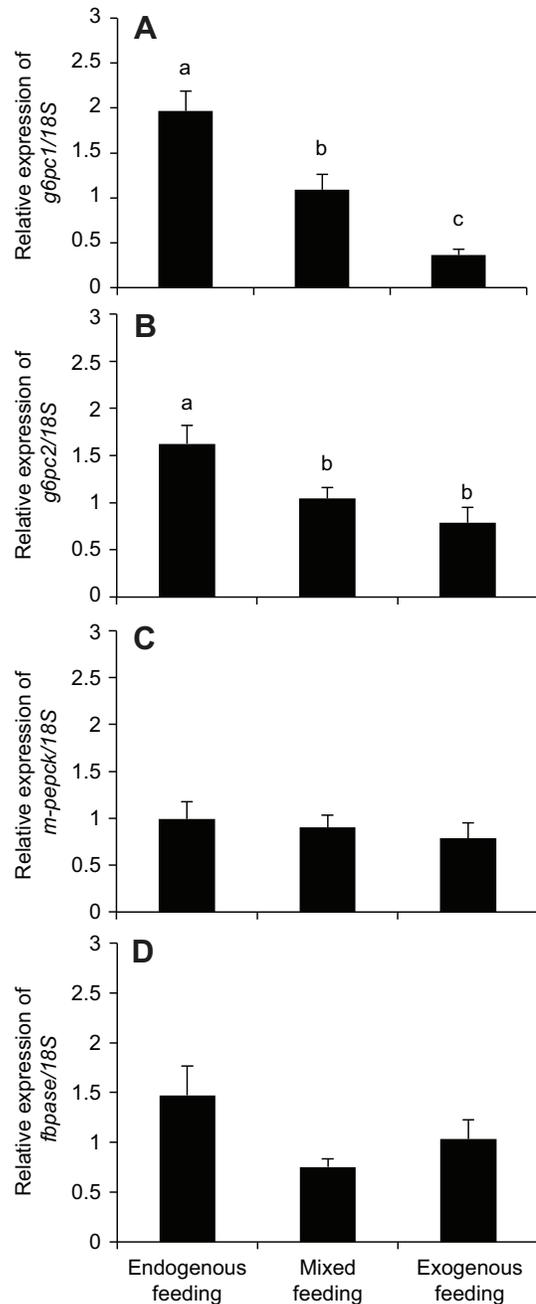


Fig. 3. Whole-body gene expression of genes involved in the intermediary metabolic pathway of gluconeogenesis in alevins utilizing different nutritional strategies. Mean  $\pm$  s.e.m. gene expression data ( $N=9$ ) for (A) glucose-6-phosphatase 1, (B) glucose-6-phosphatase 2, (C) fructose-bisphosphatase and (D) phospho-*enol*pyruvate-carboxykinase are shown. Different letters indicate significantly different groups as established by one-way ANOVA and Student–Newman–Keuls *post hoc* test.

(Fig. 2B,C). Conversely, the glucose-6-phosphatase genes coding the last enzyme in glucose release 1, *g6pc1* (d.f.=2,  $F=29.466$ ,  $P<0.01$ ), and glucose-6-phosphatase 2, *g6pc2* (d.f.=2,  $F=8.27$ ,  $P<0.01$ ), were significantly decreased in extroph alevins (Fig. 3A,B). No changes were found in the expression of the gluconeogenic genes mitochondrial phosphoenolpyruvate carboxykinase, *m-pepck* (d.f.=2,  $F=1.534$ ,  $P>0.05$ ), or fructose-1,6 bisphosphatase 1, *fbpase1* (d.f.=2,  $F=2.988$ ,  $P>0.05$ ), between

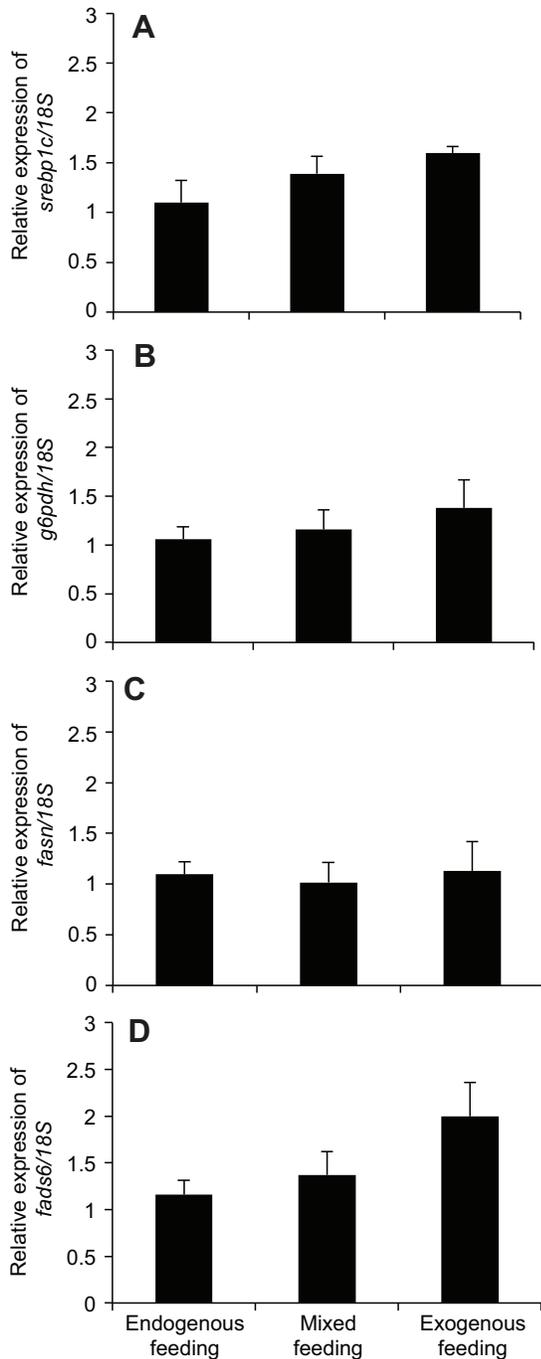


Fig. 4. Whole-body gene expression of genes involved in the intermediary metabolic pathway of lipogenesis and lipid metabolism in alevins utilizing different nutritional strategies. Mean  $\pm$  s.e.m. gene expression data ( $N=9$ ) for (A) sterol regulatory binding protein 1c, (B) glucose-6-phosphate dehydrogenase, (C) fatty acid synthase and (D) fatty acid delta6 desaturase are shown.

rainbow trout alevins using different nutritional sources (Fig. 3C,D). The expression of gene markers for lipid metabolism were also investigated; no significant changes were observed in the expression of the lipogenic genes (Fig. 4A–C) sterol regulatory element binding protein 1c, *srebp1c* (d.f.=2,  $F=1.617$ ,  $P>0.05$ ), fatty acid synthase, *fasn* (d.f.=2,  $F=0.98$ ,  $P>0.05$ ), or glucose-6-phosphate dehydrogenase, *g6pdh*, which functions as an electron donor in the

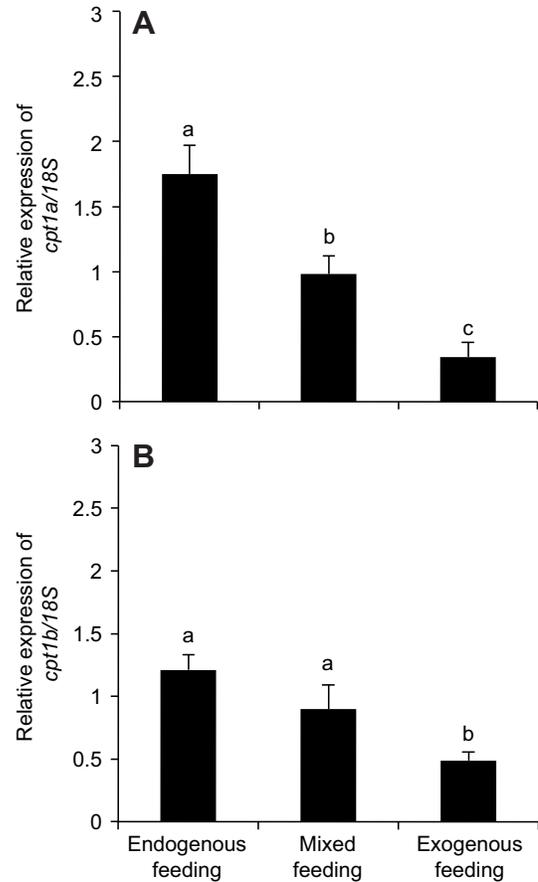


Fig. 5. Whole-body gene expression of genes involved in the intermediary metabolic pathway of fatty acid  $\beta$ -oxidation in alevins utilizing different nutritional strategies. Mean  $\pm$  s.e.m. gene expression data ( $N=9$ ) for (A) carnitine palmitoyl transferase 1A and (B) carnitine palmitoyl transferase 1B are shown. Different letters indicate significantly different groups as established by one-way ANOVA and Student–Newman–Keuls *post hoc* test.

lipogenic pathway (d.f.=2,  $F=1.450$ ,  $P>0.05$ ). Furthermore, no changes were observed in fatty acid desaturase 2, *fads2* (d.f.=2,  $F=1.852$ ,  $P>0.05$ ; Fig. 4D). In contrast, a significant decrease in the expression of genes involved in  $\beta$ -oxidation pathways, particularly the isoforms of the rate-limiting carnitine-palmitoyl transferase transporter, *cpt1a* (d.f.=2,  $F=30.15$ ,  $P<0.01$ ) and *cpt1b* (d.f.=2,  $F=10.86$ ,  $P<0.01$ ), was observed in fish relying on exogenous feeding (Fig. 5). The gene expression of the enzymes serine dehydratase, *sdh* (d.f.=2,  $F=3.176$ ,  $P>0.05$ ), arginine synthase, *as* (d.f.=2,  $F=1.592$ ,  $P>0.05$ ), and glutamine synthase, *gls* ( $F=1.329$ ,  $P>0.05$ ), and the glutamate dehydrogenase isoforms *gdh1* (d.f.=2,  $F=0.125$ ,  $P>0.05$ ) and *gdh2* (d.f.=2,  $F=1.22$ ,  $P>0.05$ ), involved in amino acid catabolism, did not change significantly (Fig. 6).

#### Expression of specific miRNAs and associated genes in trout alevins switching from endogenous to exogenous feeding

The expression of *omy-miRNA-33* (d.f.=2,  $F=65.7$ ,  $P<0.01$ ) and *omy-miRNA-122a/b* isomiRs (d.f.=2,  $F=8.809$  and d.f.=2,  $F=8.901$ , both  $P<0.01$ ) decreased in fish that rely exclusively on exogenous feeding (Fig. 7). The expression of *omy-miRNA-143* (d.f.=2,  $F=6.745$ ,  $P>0.01$ ) increased in fish that rely on mixed and exogenous feeding (Fig. 8A). The gene *abhd5* is a predicted direct target of *omy-miR-143* in rainbow trout (Salem et al., 2010). The *omy-miRNA-*

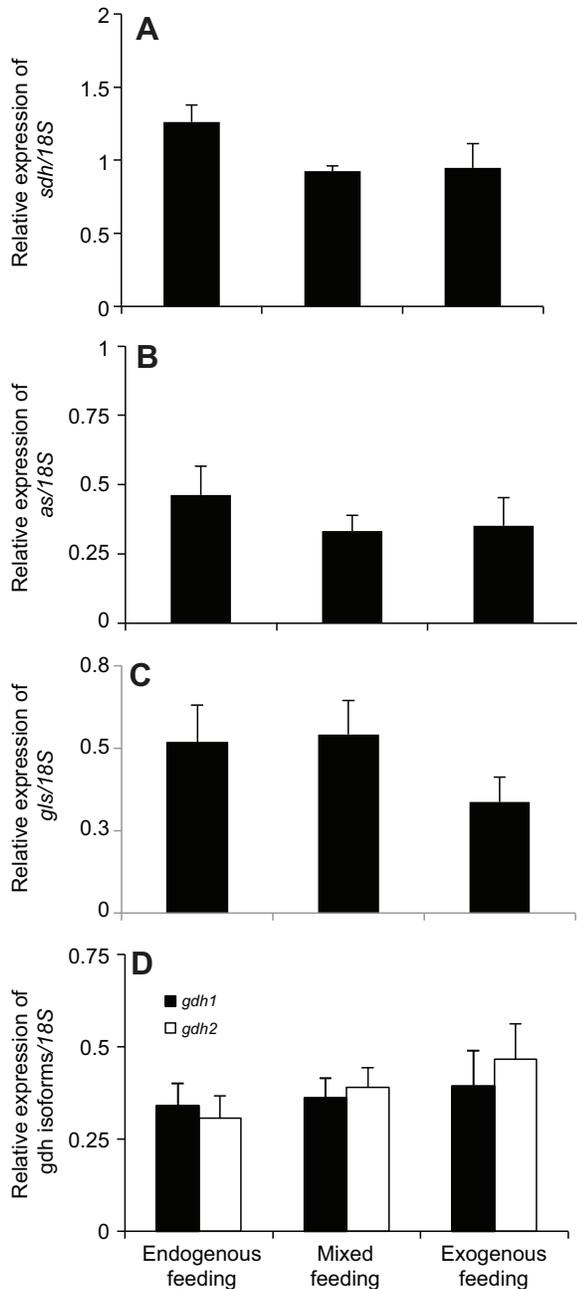


Fig. 6. Whole-body gene expression of genes involved in the intermediary metabolic pathway of amino acid catabolism in alevins utilizing different nutritional strategies. Mean  $\pm$  s.e.m. gene expression data ( $N=9$ ) for (A) serine dehydratase, (B) alanine synthase, (C) glutamine synthase and (D) serine dehydratase isoforms are shown.

143 was predicted to bind to multiple (six) target sites in the mRNA for *abhd5* in trout. To further assess the prediction of the trout *abhd5* homologue as a target of *omy-miRNA-143* in trout, we mapped several of these binding sites onto the available sequence in order to obtain additional information with respect to the localization of the binding sites, as the presence of binding sites in the 3' UTR results in comparatively stronger expression than target sites in the coding sequences. In cases where target sites are present in both coding sequences and the 3' UTR, such as the case of *omy-miRNA-143* binding sites in trout, a mild but significantly enhanced

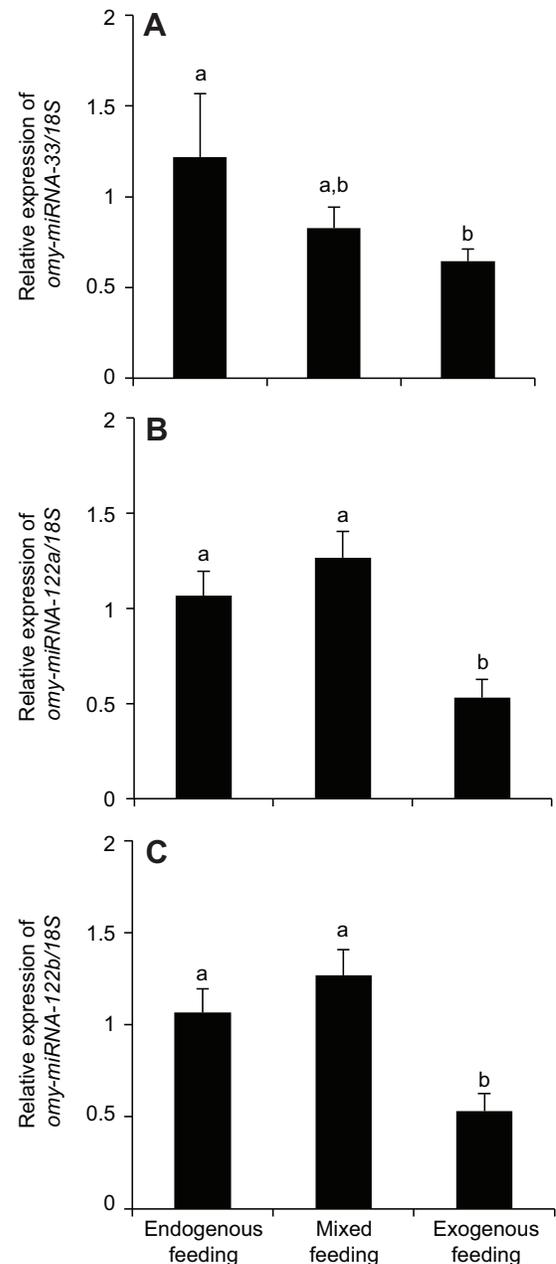


Fig. 7. Whole-body gene expression of liver-specific *omy-miRNA* in alevins using different nutritional strategies. Mean  $\pm$  s.e.m. gene expression data ( $N=9$ ) for (A) *omy-miRNA-33*, (B) *omy-miRNA-122a* and (C) *omy-miRNA-122b* are shown. Different letters indicate significantly different groups as established by one-way ANOVA and Student–Newman–Keuls *post hoc* test.

repression is generally observed (Fang and Rajewsky, 2011). The novel information that indeed two of these potential binding sequences were located in the predicted 3' UTR of the *abhd5* transcript in rainbow trout (supplementary material Fig. S1), while three additional target sequences are located within the coding sequence of the mRNA, further strengthens the case for a role of *omy-miRNA-143* in the regulation of trout *abhd5*. The expression of *abhd5* (Fig. 8B) decreased with the acquisition of an exogenous feeding strategy in whole alevins (d.f.=2,  $F=10.619$ ,  $P<0.05$ ) and is furthermore inversely correlated with the expression of its

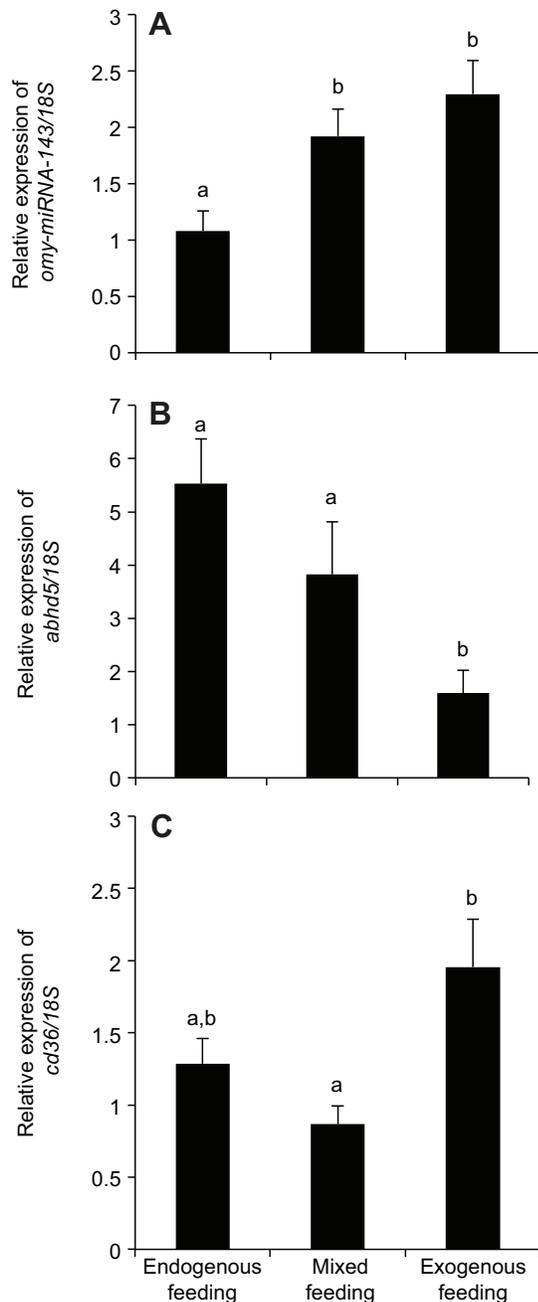


Fig. 8. Whole-body alevin gene expression of (A) adipose tissue-enriched *omy-miRNA-143*, (B) its predicted direct target *abhd5* and (C) the adipogenesis marker *cd36* in alevins using different nutritional strategies. The expression data are means  $\pm$  s.e.m. ( $N=9$ ). Different letters indicate significantly different groups as established by one-way ANOVA and Student–Newman–Keuls *post hoc* test.

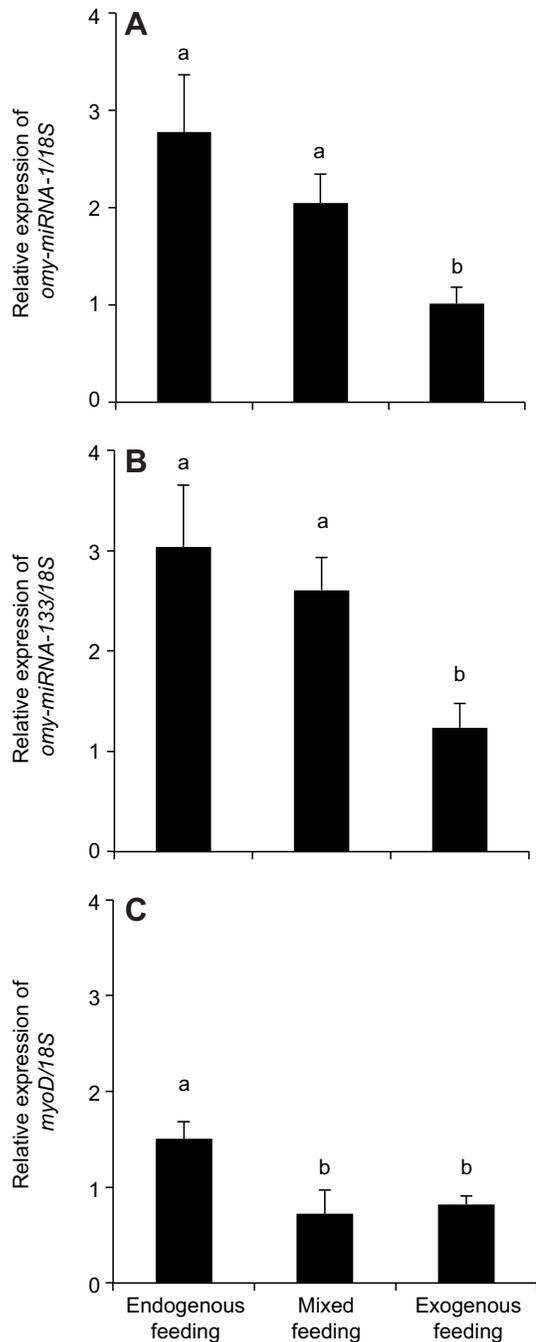


Fig. 9. Whole-body gene expression of muscle-specific *omy-miRNA-1* (A) and *omy-miRNA-133* (B), as well as myocyte-specific transcription factor *myoD* (C) in alevins using different nutritional strategies. The expression data are means  $\pm$  s.e.m. ( $N=9$ ). Different letters indicate significantly different groups as established by one-way ANOVA and Student–Newman–Keuls *post hoc* test.

predicted regulatory miRNA, *omy-miRNA-143*, as assessed by Spearman's test ( $\sigma=0.514$ ,  $P<0.05$ ). The expression of *cd36*, a marker of adipogenesis in mammalian models, was used to validate adipogenic potential in trout alevins at a molecular level and was increased in exogenously feeding alevins compared with alevins at first feeding (d.f.=2,  $F=5.13$ ,  $P<0.05$ ; Fig. 8C). The expression of muscle-specific *omy-miRNA-1* (d.f.=2,  $F=4.75$ ,  $P<0.05$ ) and *omy-miRNA-133* (d.f.=2,  $F=5.301$ ,  $P<0.05$ ) decreased significantly in

alevins that fed exogenously (Fig. 9A,B). The expression of *omy-miRNA-1* and *omy-miRNA-133* was significantly correlated ( $\sigma=0.558$ ,  $P<0.01$ ), and hence the expression of *myoD*, a stimulatory transcription factor for the miR-1/133 locus in mammals, was investigated. In spite of a concurrent decrease in *myoD* expression in exogenously feeding alevins (d.f.=2,  $F=4.068$ ,  $P<0.05$ ; Fig. 9C), no significant correlations between the expression of *myoD* and *omy-miRNA-1* ( $\sigma=0.219$ ,  $P>0.05$ ) or *myoD* and *omy-miRNA-133*

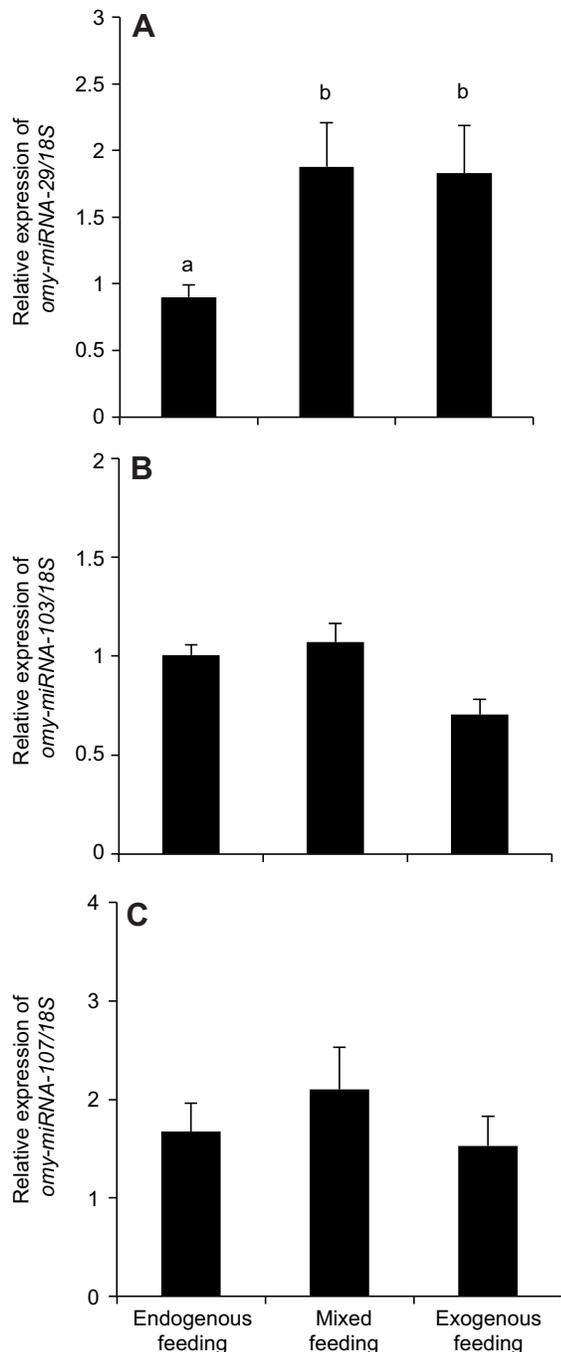


Fig. 10. Whole-body gene expression of ubiquitously expressed omy-miRNA genes putatively involved in the regulation of intermediary metabolism in alevins using different nutritional strategies. Global alevin gene expression data (means  $\pm$  s.e.m.;  $N=9$ ) of (A) *omy-miRNA-29*, (B) *omy-miRNA-103* and (C) *omy-miRNA-107* are shown. Different letters indicate significantly different groups as established by one-way ANOVA and Student–Newman–Keuls *post hoc* test.

( $\sigma=0.158$ ,  $P>0.05$ ) were found. The expression of the ubiquitously expressed *omy-miRNA-29* (d.f.=2,  $F=4.475$ ,  $P<0.05$ ; Fig. 10A) increased significantly in fish relying on exogenous feeding, while the ubiquitously expressed *omy-miRNA-103/107* (d.f.=2,  $F=2.44$ ,  $P>0.05$  and d.f.=2,  $F=0.81$ ,  $P>0.05$ ) did not change significantly between fish relying on different feed sources (Fig. 10B,C).

## DISCUSSION

### Global inhibition of $\beta$ -oxidation and glucose production pathways in alevins utilizing an exogenous diet

Globally, gene expression analysis revealed a progressive decrease in the expression of genes involved in the fatty acid  $\beta$ -oxidation and in the last step of glucose release, as evidenced by the significantly decreased expression of the analyzed *cpta* and *cptb* isoforms and *g6pc1* and *g6pc2* isoforms, respectively. This gene expression profile is in line with the hypothesized change in metabolic strategy in oviparous fish larvae, which initially rely on endogenous reserves of lipids and proteins, believed to contribute to the maintenance energy metabolism by these catabolic pathways (Desrosiers et al., 2008; Boulekbache, 1981; Conceição et al., 1998; Ronnestad et al., 1999). The initial high expression of *cpt* gene isoforms, generally considered to represent the rate-limiting step in the  $\beta$ -oxidation pathway of free fatty acids by regulating their mitochondrial uptake, and the subsequent decrease in *cpt* isoform expression at the onset of exogenous feeding, are also in accordance with the detection of active lipid turnover in trout embryos (Termer et al., 1968), believed to liberate free fatty acids from the principally stored phospholipids and triglycerides in order to provide a supply for oxidation in order to ensure continuous supply of energy (Wiegand, 1996; Termer et al., 1968; Tocher, 2003). Indeed, due to a very small glycogen storage capacity, which is consumed very quickly at the beginning of development, and the comparatively very high quantity of vitelline lipid and protein content (Termer et al., 1968), trout alevins are believed to use triglyceride-derived glycerol, but also gluconeogenic amino acids as substrates for glucose synthesis (Termer et al., 1968). Therefore, even though the expression of two other specific gluconeogenic enzymes *pepck* and *fbpase* did not change, the similarly initial high expression of *g6pc* isoforms and their decrease in expression after alevins switch to exogenous feeding seems to be equally in concordance with this hypothesized production of glucose in trout alevins. The initially detected higher expression in *g6pase* isoforms in trout alevins is also consistent with the evidence for G6Pase activity in embryonic rainbow trout, which increases slightly up to first feeding (Vernier and Sire, 1976). Conversely, *gk* gene expression increased following the first exogenous feed intake, confirming previous findings in trout (Geurden et al., 2007) and carp (Panserat et al., 2001). The induction of the expression of the gene *gk*, involved in the first step of glucose use, and concurrent decrease in the expression of *g6pc* are furthermore consistent with findings from a transcriptomic analysis of complete European sea bass (*Dicentrus labrax*) larvae, in which several genes that are involved in the glycolytic pathway are increasingly expressed with advancing ontogenesis, while genes involved in gluconeogenesis exhibit an inverse expression pattern, with initially highly expressed genes that subsequently decrease (Darias et al., 2008). Moreover, even though there is induction of *gk* gene expression after feeding, this induction is relatively low (threefold) compared with the effects of carbohydrate-rich diets [up to 200-fold (Seiliez et al., 2011)]. This relatively low *gk* induction is probably linked to the low level of carbohydrates in the starter diet (coming only from the 13.5% corn starch). The low *gk* induction could also explain the absence of induction of *g6pdh*, *fas* and *srebp1c* gene expression, the lipogenesis being induced when the glucose is in excess. However, this induction of *gk* gene expression indicates unambiguously that there was glucose intake in alevins fed a diet that could be sufficient for a concomitant decrease of lipid  $\beta$ -oxydation (as reflected by the *cpt1* gene expression). Therefore, the ontogenetic switch between nutritional phenotypes in rainbow trout may at least partially be regulated at

the level of gene expression in a pattern that is consistent with the hypothesized switch from the utilization of yolk reserves to external feeding, and may indeed be common feature in different oviparous fish that undergo ontogenetic changes in nutritional phenotypes.

#### Ontogenic changes in ubiquitously expressed miRNAs with metabolic function

Several miRNAs with metabolic function have been characterized in mammals and have been shown to functionally regulate carbohydrate and/or lipid metabolism (Rottiers and Näär, 2012). For example, miRNA-29, miRNA-103 and miRNA-107 have been shown to inhibit insulin-induced Akt signaling, in effect stimulating gluconeogenic gene expression in the liver with consequences for glucose homeostasis in mammalian models (Pandey et al., 2011; Trajkovski et al., 2011; Jordan et al., 2011). While direct mechanistic evidence for similar roles in fish is still lacking, correlational descriptive studies reveal a possible conservation of these functions in fish (Her et al., 2011; Mennigen et al., 2012). In our study, no changes were observed in the expression of miR-103/107 in trout using different feeding strategies in spite of an evident regulation of some gluconeogenic genes. Because these microRNAs are in contrast to liver-, adipose- and muscle-enriched miRNAs ubiquitously expressed in adult trout metabolic tissues, this result is difficult to interpret, as it may reflect a differential regulation of these miRNAs in different tissues, as is the case in mammals (Trajkovski et al., 2011). While not many ontogenetic profiles for miRNA expression in fish are currently available that provide data for first feeding, miR103/107 abundance increases temporally with first feeding in Atlantic halibut (Bizuyehu et al., 2012), contrary to the results obtained in our study. Interestingly, *omy-miRNA-29* increased significantly with first feeding and remained elevated in exogenously feeding fish. The *rno-miRNA-29* has been shown to be highly upregulated in liver, adipose tissue and muscle of diabetic rats (He et al., 2007) and has hence been shown to have metabolic functions *via* modulation of the insulin pathway in the liver (Pandey et al., 2011).

#### Decreased expression of liver-enriched *omy-miRNA-33* and *omy-miRNA-122* in exogenously feeding rainbow trout

A concurrent decrease in *omy-miRNA-33* and *omy-miRNA-122* (primarily expressed in the liver in adult trout; data not shown) was observed in rainbow trout relying on completely exogenous feeding. The decreased expression of both miRNAs known to inhibit hepatocyte proliferation in higher vertebrates (Xu et al., 2010; Cirera-Salinas et al., 2012) may be also be related to the relatively higher allometric contribution of liver growth compared with other tissues in rainbow trout fingerlings (Weatherley and Gill, 1983), indicating increased proliferation of hepatic tissue at this stage. As both miRNA-122 and miRNA-33 are known to inhibit hepatocyte proliferation, their expression decrease in exogenously feeding alevins may be related to the increased proliferation of hepatic tissue observed in rainbow trout alevins and juveniles, in line with increased metabolic demands in the growing alevins. Nevertheless, while genes with a function in cell proliferation are predicted to be targeted in fish species whose genome sequences are available from the Microcosm algorithms ([www.ebi.ac.uk/enright-srv/microcosm/](http://www.ebi.ac.uk/enright-srv/microcosm/)), direct proof in fish is lacking to date and these hypotheses remain to be validated in fish. In rainbow trout, whose genome has not fully been sequenced and annotated, no targets for miR-122 have yet been predicted. Compared with the developmental profile of miR-122 in other fish species, the observed pattern is different from changes following first feeding in Atlantic halibut (Bizuyehu et

al., 2012), where miRNA-122 abundance increases sharply at first feeding before decreasing in early metamorphosis, indicating species-specific differences in ontogenetic miRNA expression profiles.

#### Increased expression of *omy-miRNA-143* as a potential functional marker for lipid deposition in rainbow trout

The identification of a particularly high expression of *omy-miRNA-143* adipose tissue in adult rainbow trout (data not shown) is in line with its established role of its human (Esau et al., 2004), chicken (Wang et al., 2012), murine (Takanabe et al., 2008) and porcine (Li et al., 2011) homologues, whose increase in adipocyte precursor cells is a necessary factor in adipocyte differentiation, at least partially through the mediation of enhanced accumulation of triglycerides (Wang et al., 2012). In a previous study, *omy-miRNA-143* was found to be mostly expressed in trout muscle and digestive tissues; however, adipose tissue was not included in the tissues analyzed (Salem et al., 2010). Both muscle and intestinal tissue are known to contain adipose tissue (Johnston and Moon, 2001; Fauconneau et al., 1997; Weil et al., 2009) and may thus have contributed to the detection of *omy-miR-143* expression in these tissues. In fish development, the process of adipogenesis is only scarcely characterized, but has clearly been demonstrated to commence with exogenous feeding in zebrafish (Flynn et al., 2009), after which the appearance of visceral adipocytes indicates a storage of excess ingested energy in form of lipids. Similarly, in rainbow trout, adipocyte number and diameter increase in juvenile trout muscle with age (Fauconneau et al., 1997). Indeed, in our study an increase in *omy-miRNA-143* was observed with the beginning of exogenous feeding, which correlates with previously reported findings that showed a significant increase in whole alevin lipid content as early as 10–30 days after the first meal (F. Médale, unpublished data). It is also known that visceral fat deposition has a high positive allometric contribution in young trout fingerlings (Weatherley and Gill, 1983). In spite of the fact that the direct localization of *omy-miRNA-143* expression can only be assessed by *in situ* analysis, the observation of a potent induction of *omy-miRNA-143* at first feeding and its strong enrichment in adipose tissue in adult rainbow trout suggest that *omy-miRNA-143* may constitute a marker for adipogenesis in rainbow trout. In contrast to other miRNAs investigated in this study, Salem et al. (Salem et al., 2010), and our own more detailed analysis, identified in *abhd5* a putative target for *omy-miRNA-143*. In addition to being a hypothesized target of *omy-miRNA-143* which is enriched in adipose tissues, the function of *abhd5* in mammalian model species is interesting, as the *abhd5* gene is involved in adipocyte degradation and liberation of free fatty acids from stored triglycerides in mammals (Lass et al., 2006), principally by acting as a strong, positive regulator of the adipose triglyceride lipase (Lass et al., 2006; Yen and Farese, 2006; Wang et al., 2012). Recently, a similar function for *abhd5* has been established in skeletal muscle, where its overexpression induces a twofold increase in triacylglycerol hydrolysis, triacylglycerol-derived fatty acid release and fatty acid oxidation, while *abhd5* knock-down reveals opposite effects (Badin et al., 2012). To investigate a possible regulatory role of *omy-miRNA-143* on *abhd5* expression, we measured both expression levels across feeding stages and identified a significant inverse correlation in the expression of *omy-miR-143* and *abhd5*. The significant increase in expression of *omy-miRNA-143* following postprandial feeding may therefore represent a functional marker in lipid deposition, accompanied by an inverse expression of its predicted target in trout, *abhd5*. Alevins that develop towards an exogenous nutritional may

thus, through this molecular pathway, favour deposition of lipids, which has been observed in trout alevins following first feeding (F.M., unpublished data) and in the muscle of juvenile trout (Fauconneau et al., 1997). In adult trout, these stores will later fulfill metabolic demands during fasting (Jeziarska et al., 1982) and exercise (Richards et al., 2002), and also play a role in cold acclimatization (Egginton et al., 2000). In order to substantiate the potential role of *omy-miRNA-143* as a potential marker for the process of lipid deposition during ontogenesis, we equally investigated the expression of *cd36*, a fatty acid transporter with a rate-limiting function on triglyceride synthesis in adipocytes and muscle (Coburn et al., 2001; Christiaens et al., 2012), which in rainbow trout has been identified as a marker for muscular lipid deposition (Kolditz et al., 2010). In our study, we found an expression level that significantly increased between first feeding and completely exogenously feeding alevins. This molecular signature is indicative of a concurrent enhanced capacity for uptake of fatty acids used for lipid deposition via *cd36*. Together, the expressions of these molecular markers indicate an increased potential for lipid deposition through increased uptake of fatty acids and decreased hydrolysis of triglycerides. This physiological switch towards lipid storage is furthermore supported by the previously described decrease in *cpt1a* and *cpt1b* expression, both markers of  $\beta$ -oxidation and involved in the functional utilization of stored lipids in the mitochondria of adult rainbow trout muscle (Morash and McClelland, 2011). However, while the expression pattern of *omy-miRNA-143* and *abhd5* supports the hypothesis of a functional role of decreased lipolysis in the switch towards storage of lipids in exogenously feeding trout alevins, a limitation of the present study is the fact that entire alevins were investigated. While *omy-miRNA-143* is indeed strongly enriched in adipose tissue of adult rainbow trout (data not shown), *in situ* hybridizations of *omy-miRNA-143* and *abhd5* in trout development will be necessary to confirm tissue specificity of *omy-miRNA-143* in trout adipose tissue during ontogenesis. Adequate methods for whole-mount *in situ* detection of small miRNAs in fish have recently become available (Legendijk et al., 2012). Additionally, only *in vitro* studies of trout adipocytes (Albalat et al., 2005) will be able to directly validate a role for *omy-miRNA-143* in lipid deposition in adipose tissue in rainbow trout, as a functional role of *omy-miRNA-143* can be unequivocally shown only by direct inhibition.

**The *omy-miR 1/133* expression pattern is indicative of a hypertrophic response in muscle in trout alevins following the transition to exogenous feeding**

The role of the muscle-specific miRNA-1 and miRNA-133 (Salem et al., 2010), found to decrease in rainbow trout alevins adopting an exogenous nutritional strategy in the present study, is generally considered to be the coordination of the balance between muscle proliferation and differentiation in mammals (Chen et al., 2006). Additionally, a role for miRNA-1 and miRNA-133 in muscle hypertrophy has been postulated based on an observed concurrent downregulation of both miRNAs in rat muscle undergoing hypertrophy following functional overload (McCarthy and Esser, 2007). Indeed, immediately prior to the first meal, trout alevins hatch and distinctly increase foraging as they begin swimming upwards in the water column (Hale, 1999). This increase in the utilization of muscles may similarly induce hypertrophy as a response. Furthermore, hypertrophy, in addition to hyperplasia, plays an important role in the continuous muscle growth in rainbow trout. The contribution of hypertrophy is indeed highest (40%) in trout alevins comparable to the stages analyzed in the present study

(Mommsen, 2001; Valente et al., 1999). Similarly, while the differentiation of muscles occurs early and is largely differentiated pre-hatching in rainbow trout (Rescan et al., 2001), an expansion of somite size and width by more than fourfold around the time of hatching and yolk resorption is observed (Bobe et al., 2000). It has furthermore been postulated in some studies that in adult trout, hypertrophy becomes the predominant mode of muscle growth (Stickland, 1983). Therefore, the observed pattern of decreased *omy-miRNA-1* and *omy-miRNA-133* may, as in mammals, be indicative of hypertrophy. However, future studies are necessary to confirm this hypothesis. Functionally, no direct targets for either *omy-miRNA-1* or *omy-miRNA-133* have been predicted by Salem and colleagues (Salem et al., 2010), so the validation of direct targets is difficult. Nevertheless, two recent studies in fish provide evidence for a conserved role for both miRNA-1 and miRNA-133s in fish muscle development. In zebrafish, homologous muscle-specific *dre-miRNA-1* and *dre-miRNA-133* have been experimentally shown to account for 50% of miRNA-dependent gene regulation in muscle (Mishima et al., 2009). These genes are enriched for actin-related genes, in line with a functional role in sarcomeric actin organization shown in the same study (Mishima et al., 2009). Additionally, antagomiR injection studies inhibiting expression of *oni-miRNA-1* and *oni-miRNA-133* in Nile tilapia reveal that specific myogenic factors are regulated in the same fashion as in mammals (Yan et al., 2012). Therefore, we investigated the expression of *myod1*, which in mammals positively regulates the expression of the miRNA-1/133 locus (Rao et al., 2006; Hirai et al., 2010). While we indeed found a concurrently decreased expression of *myoD1* in exogenously feeding trout alevins, no significant correlation in the expression was found, implicating that the action of *myoD* on this cluster may not be conserved or that the regulation of *omy-miRNA-1* and *omy-miRNA-133* is complex and not principally mediated by *myod1*, or that the expression of *myoD1* is not indicative of the abundance and/or activity of *myoD*. Similar to the results in our study, no direct relationship in the ontogenic expression of *myoD* and miRNA-1/133 has been reported in the ontogenesis of the flounder *Paralichthys olivaceus* (Xie et al., 2011; Fu et al., 2012). In the characterization of developmental miRNA expression in Atlantic halibut, the abundance of *hhi-miRNA-133a/b* equally decreased significantly following first feeding (Bizuyehu et al., 2012). This indicates that nutritional factors may play a role in the regulation of miRNA-1 and miRNA-133. The finding that in fruitfly (*Drosophila melanogaster*) larvae miRNA-1 is critically required for muscle growth only following first feeding, but not prior to this event (Sokol and Ambros, 2005), supports this interpretation. Overall, *omy-miRNA-1* and *omy-miRNA-133* may represent markers for specific forms of trout muscle development, and could be regulated both by increased foraging and exogenous nutrition. Further studies in trout are necessary to confirm a conserved role of both *omy-miRNA-1* and *omy-miRNA-133* between trout and mammals, and progress in the sequencing and annotation of the trout genome will greatly facilitate this task.

**Conclusions**

The results presented in this paper reveal underlying molecular changes in both gene expression and miRNA expression in trout alevins that switch from an endogenous to an exogenous feeding strategy. The global gene expression pattern of gene markers of metabolic pathways is consistent with a switch from utilization of protein and lipid reserves typically found in yolk to the utilization and subsequent storage of external feeds. The molecular signature is consistent with increased lipid storage through increased uptake

of fatty acids and decreased utilization and is in line with the fact that lipid content in rainbow trout is considered to be highly sensitive to exogenous stimuli, including nutrition (Dumas et al., 2007). The observed expression of adipose-enriched miRNA-143 and the inverse decrease in the expression of its predicted target *abhd5*, involved in lipolysis of triglycerides in adipocytes, are consistent with this profile and suggest that miRNAs may play an important role in the ontogenetic regulation of gene expression that underlies the change in nutritional source. However, the sequencing and annotation of the trout genome, as well as the functional study of this and other miRNAs in trout tissues, will be necessary to fully establish these miRNAs as markers of metabolic tissue function.

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### AUTHOR CONTRIBUTIONS

Conception and design of the study: J.A.M., S.S. and S.P. Execution of the study: J.A.M. and S.S. Interpretation of the findings published: J.A.M., S.S. and S.P. Drafting of the article: J.A.M. Revision of the article: S.S. and S.P.

### COMPETING INTERESTS

No competing interests declared.

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