

1 **Kinetic study of the expression of genes related to hepatic steatosis, global intermediate**
2 **metabolism and cellular stress during overfeeding in mule ducks**

3
4 Tracy Pioche¹, Fabien Skiba², Marie-Dominique Bernadet³, Iban Seiliez¹, William
5 Massimino¹, Marianne Houssier¹, Annabelle Tavernier¹, Karine Ricaud¹, Stéphane Davail¹,
6 Sandrine Skiba-Cassy¹, Karine Gontier¹

7
8 ¹INRA, Univ Pau & Pays Adour, E2S UPPA, UMR 1419, Nutrition, Métabolisme,
9 Aquaculture, Saint-Pée-sur-Nivelle, F-64310, France

10
11 ²NUTRICIA, Route de Saint-Sever, F-40280 Haut-Mauco, France

12
13 ³UEPFG INRA Bordeaux-Aquitaine, (Unité Expérimentale Palmipèdes à Foie Gras), Domaine
14 d'Artiguères 1076, route de Haut-Mauco, F-40280 Benquet, France

15
16 Corresponding author: Gontier Karine

17 INRA, Univ Pau & Pays Adour, E2S UPPA, UMR 1419, Nutrition, Métabolisme, Aquaculture,
18 Saint-Pée-sur-Nivelle, F-64310, France

19 e-mail: karine.gontier@univ-pau.fr; tel.: +33 558 513 706; fax: +33 558 513 737

20
21 **Keywords:**

22 Hepatic steatosis, lipogenesis, cholesterol, cellular stress, mule ducks

23
24 **ABSTRACT**

25
26 Induced by overfeeding, hepatic steatosis is a reversible process exploited for “foie gras”
27 production. To better understand the mechanisms underlying this non-pathological
28 phenomenon, we analysed the physiological responses of the mule duck to cope with 22
29 carbohydrate meals. A kinetic analysis of intermediate metabolism and cell protection
30 mechanisms was performed during overfeeding. As expected, dietary carbohydrates are up
31 taken mainly by the liver (*chrebp*, *glut1/2/8*) and converted into lipids (*acox*, *scd1*, *acs11*, *fas*,
32 *dgat2*). Our study showed an activation of cholesterol biosynthetic pathway with significant
33 correlations between plasma cholesterol, expression of key genes (*hmgcr*, *soat1*) and liver
34 weight. Results revealed an activation of insulin and amino acid cell signalling pathway

35 suggesting that ducks boost insulin sensitivity to raise glucose uptake and use *via* glycolysis
36 and lipogenesis. Expression of *cpt1a*, *acad*, *hadh* suggested an induction of beta-oxidation
37 probably to remove part of newly synthesized lipids and avoid lipotoxicity. Cellular stress
38 analysis revealed an upregulation of autophagy-related gene expression (*atg8*, *atg9*, *sqstm1*) in
39 contrast with an induction of *cyp2e1* suggesting that autophagy could be suppressed. *Lamp2a*
40 and *plin2* enhanced, conflicting with the idea of an inhibition of lipophagy. *Hsbp1*
41 overexpression indicated that mechanisms are carried out during overfeeding to limit cellular
42 stress and apoptosis to prevent the switch to pathological state. *Atf4* and *asns* overexpression
43 reflects the nutritional imbalance during overfeeding. These results permitted to highlight the
44 mechanisms enabling mule ducks to efficiently handle the huge starch overload and reveal
45 potential biomarker candidates of hepatic steatosis as plasma cholesterol for liver weight.

46 **INTRODUCTION**

47

48 Hepatic steatosis (also called fatty liver or “foie gras”) is induced by overfeeding (21). This
49 phenomenon is reversible and allow liver to return to its initial composition when overfeeding
50 is interrupted (4, 30). This process, that spontaneously occurs in some birds as a consequence
51 of energy storage before migration (37), is exploited today for the “foie gras” production (20).
52 The metabolic response to overfeeding is extremely variable and depends on the genotype of
53 ducks (48). Mule duck, a sterile hybrid resulting from the crossing between a male Muscovy
54 (*Cairina moschata*) and a female Pekin duck (common duck, *Anas platyrhynchos*), is preferred
55 for fatty liver production (6, 20). Indeed, it benefits from a heterosis effect and shares qualities
56 specific to each of its parents, in particular a superior ingestion capacity as well as a bigger
57 “foie gras” (14).

58

59 Various mechanisms are known to take place during the establishment of hepatic steatosis.
60 Firstly, the carbohydrate-rich corn based diet received by the animals during overfeeding leads
61 up to high lipid accumulation as a result of high activity of *de novo* lipogenesis, mostly in the
62 liver (11), but also to a substantial fattening of peripheral tissues such as adipose tissue and
63 muscles (48). Then, to contribute to lipid accumulation in the liver, studies have highlighted a
64 combination of three mechanisms: a defect in lipid exports from liver to peripheral tissues, a
65 lack of lipid capture by the peripheral tissues and a significant return of lipids to liver (48, 49).

66

67 Others mechanisms and cellular pathways may be involved in the development of fatty liver.
68 Among these, we cannot ignore the insulin pathway who is strongly involved in the storage of
69 carbohydrate and lipid substrates and little studied during overfeeding. As a well-known
70 anabolic hormone, insulin is involved in the regulation of carbohydrates and lipids metabolism.
71 Insulin controls the transport of glucose in insulin-dependent tissues (adipose and muscle
72 tissues) and stimulates glycolysis, glyconeogenesis and lipogenesis. It promotes the synthesis
73 and the storage of carbohydrates and lipids participating thus in their homeostasis (3, 29, 42).
74 Insulin also has the ability to stimulate protein synthesis and inhibit the mechanisms of cell
75 degradation via activation of the mTOR pathway. It seems therefore relevant to evaluate the
76 role of this signalling pathway in the development of hepatic steatosis in mule ducks.

77

78 Among other factors, we also wanted to study the metabolism of cholesterol, especially for an
79 important aspect: its involvement in membrane fluidity (10, 47) that could influence the melting

80 rate, a parameter strongly involved in the technological yield of fatty liver. In fact, cholesterol,
81 which is essential for cell growth and viability, is known to enter into the constitution of cell
82 membranes and the synthesis of steroid hormones (55). Its synthesis is mainly hepatic and made
83 from hydroxy-methyl-glutaryl-CoA (HMG-CoA). Studies highlighted the interesting potential
84 of mule duck to produce abundant free cholesterol content at the end of the overfeeding (22,
85 41). We therefore propose to study the metabolic pathway controlling cholesterol homeostasis
86 during the development of hepatic steatosis during overfeeding in mule ducks and evaluate its
87 potential relation with melting rate.

88

89 Finally, it would be interesting to study the mechanisms involved to understand how overfed
90 ducks can manage such accumulation of lipids in the liver without apparent lipotoxicity. In fact,
91 oxidative stress, lipotoxicity, and inflammation have been shown to play a key role in the
92 progression of NAFLD to Non-Alcoholic Steatohepatitis (NASH) (53). Since hepatic steatosis
93 is non-pathological and reversible in ducks (4), it seemed important to study these mechanisms
94 in order to understand the protective mechanisms put in place by overfed ducks, to prevent a
95 pathological evolution of the hepatic steatosis. Autophagy is a cellular catabolic process
96 degrading of cytoplasmic components to provide nutrients at critical situations. As part of the
97 autophagic process, lipophagy is defined as the autophagic degradation of intracellular lipid
98 droplets. In murine models, inhibition of autophagy promotes lipid accumulation in the liver
99 (44) as well as endoplasmic reticulum stress and apoptosis (13). Whether these mechanisms
100 also occur during the development of hepatic steatosis in overfed mule duck remains to be
101 established.

102

103 Therefore, the aim of the present study was to better understand the mule duck physiological
104 response to cope with a sustained overload of starch during 22 consecutive meals. This will
105 provide useful information on the mechanisms underlying the development of hepatic steatosis
106 in mule duck to optimize it and propose new breeding strategies for “foie gras” production.

107

108 **MATERIALS AND METHODS**

109

110 *Animals and experimental procedures*

111 All experimental procedures were carried out according to the ethic committee (approval No.
112 C40-037-1) and the French National Guidelines for the care of animal for research purposes.

113 Animals used were male mule ducks (n=96), reared in a Louisiana type building of 80 m² with
114 2.6 ducks/m². Faced with the high risk of contamination by Influenza disease, ducks were kept
115 in complete confinement for safety. They benefited from natural lighting and were raised on
116 chip litter and watered by pipettes at the Experimental Station for Waterfowl Breeding (INRA
117 Artiguères, France). They were fed *ad libitum* with the growing diet from hatching to the age
118 of 8 weeks (17.5 MAT, 2850 Kcal) then by hourly rationing (1 h per day) from 8 to 9 weeks
119 followed by a quantitative rationing from 9 to 12 weeks in order to prepare the overfeeding
120 (15.5 MAT, 2800 Kcal). At 12 weeks of age, ducks were overfed with 22 meals (2 meals a day
121 during 11 days) composed with mash 53% MS Palma 146 from Maïsador (Maize: 98 % and
122 Premix: 2 %, 3230 Kcal, with crude protein: 7.2 %, MG: 3.2 %, crude cellulose: 2 %, raw ashes:
123 2.1 %, lysine: 0,23 %, methionine: 0.15 %, calcium: 0.12 %, phosphorus: 0.25 % and sodium:
124 0.1 %) and water. The quantity of food distributed during the overfeeding was adjusted to the
125 body weight of the animals. Two hours after the 4th (M4), the 12th (M12) and the 22th (M22)
126 meals, ducks were conventionally slaughtered by electronarcosis and bleeding. Bloods were
127 taken on EDTA tubes and plasma were separated by centrifugation (3000 xG for 10 min at 4°C)
128 and stored at -20°C. After dissection, liver, *major pectoralis* (muscle), abdominal fat (AF) and
129 subcutaneous adipose tissue (SAT) were weighed, sampled, frozen in liquid nitrogen and stored
130 at -80°C. Pasteurization tests were carried out on livers at the end of the overfeeding (M22,
131 n=32) to estimate melting rate. 4 hours after evisceration, liver samples (60 g of the top of the
132 large lobe) were placed into tins. After crimping performed, autoclave was preheated to 70°C,
133 and the tins were placed therein. As soon as the autoclave was closed, the pressure was fixed to
134 0.8 bar. Boxes were heated for 1 hour at 85°C, and then cooled for 30 min in the autoclave.
135 Then tins were stored cold until opening. Before opening, tins were warmed in a water bath and
136 then livers samples were weighed without their fat.

137

138 ***Biochemical assays***

139 Plasma glucose (GLU), triglyceride (TG) and total cholesterol (CHO) levels were quantified by
140 colorimetric enzymatic methods using kits provided by BioMérieux (Marcy-l'Etoile, France)
141 according to the manufacturer's recommendations.

142

143 ***Protein extraction and Western Blotting***

144 Frozen livers (100 mg, n=12 at each sampling time) were crushed with Precellys Cryollis
145 (Bertin Technologies, 3000 xG, 2 cycles/10 sec, break/15 sec) in 1 mL of lysis buffer (150 mM
146 NaCl, 10 mM Tris, 1 mM EGTA, 1 mM EDTA, pH 7.4), 100 mM sodium fluoride, 4 mM sodium

147 pyrophosphate, 2 mM sodium orthovanadate, 1% Triton X-100, 0.5% IGEPAL® CA-630
148 (Sigma) and a protease inhibitor cocktail (Sigma). Homogenates were centrifuged (12000 xG,
149 4°C/15 min.). The supernatants were recovered and centrifuged (12000 xG, 4°C/15 min).
150 Protein concentrations were determined by Bradford assay using BSA as standard. Lysates (10
151 µg of total protein for Akt/S6) were subjected to SDS-PAGE and western blotting using the
152 appropriate antibody. Anti-phospho-Akt (Ser 473, No. 4060), anti-Akt (No. 9272), anti-
153 phospho-S6 (Ser 235/236, No. 4856), anti-S6 (No. 2217) were purchased from Cell Signalling
154 Technologies (Ozyme, Saint Quentin Yvelines, France). All antibodies successfully cross-
155 reacted with mule duck proteins. Membranes were washed and incubated with an IRDye
156 Infrared secondary antibody (Li-COR Biosciences, Lincoln, USA). Bands were visualized by
157 infrared fluorescence and quantified by densitometry using the Odyssey Imaging System (Li-
158 COR Biosciences).

159

160

161 ***Ribonucleic acid isolation and reverse transcription***

162 *RNA extraction*

163 Total RNA were isolated from frozen tissues (liver, muscle and abdominal fat) using TRIzol
164 Reagent (Invitrogen/Life Technologies) according to manufacturer's instructions. RNA
165 concentrations were determined by spectrophotometry (optical density at 260 nm) using a
166 NanoVuePlus (GE Healthcare) and normalized at 500 ng/µL. The integrity of total RNA was
167 checked by electrophoresis (agarose gel 1 %).

168

169 *Reverse transcription*

170 After a DNase treatment using the Quanta DNase kit (Quanta Biosciences), cDNA were
171 obtained by reverse transcription using the Superscript III enzyme (Invitrogen) and a mix of
172 oligo dT and random primers (Promega) according to manufacturer's instructions. 100 pg/µL
173 of luciferase (Promega), an exogenous RNA not present in duck, was added to each sample
174 during the denaturation step to allow normalization of the data as a reference gene as previously
175 described (9, 32). 3 µg of total RNA were used. The reaction was carried out on a T100
176 thermocycler (Biorad) according to this program: 25°C / 5 min, 55°C / 1 h, 70°C / 15 min, 4°C
177 / ∞ until storage at -20°C.

178

179 ***Determination of mRNA levels using real-time PCR***

180 *qPCR EvaGreen using BioMark*

181 The Fluidigm method was used to quantify gene expression of the majority of our genes of
182 interest. For this, a specific target amplification (STA) was carried out beforehand with 5 ng/ μ L
183 of cDNA in order to normalize all the samples and to ensure that there are enough cDNA copies
184 in each well. Then, all samples and target were distributed in a 96x96 chip for Fluidigm Gene
185 Expression. The reaction was carried out using the EvaGreen 20X dye according to the
186 following program: 95°C/10 min (holding step), 35 amplification cycles (95°C/15 s, 60°C/1
187 min). All data were analysed with Fluidigm real-time PCR analysis software (Fluidigm
188 Corporation v4.1.3). This part of the work was done at the quantitative transcription platform
189 GeT-TQ (GenoToul, Toulouse, France). All the primer sequences are presented in Table 1. For
190 some genes involved in cholesterol metabolism, gene expressions were determined by qPCR
191 from 2 μ L cDNA (diluted 80 fold), target gene primers (10 μ M), SYBr Green FastMix (Quanta)
192 and RNase free water for a total volume of 15 μ L. qPCR were carried out according to the
193 following program: initiation at 95°C/10 s followed by 45 amplification cycles (60°C/15 s) on
194 the CFX Thermal cycler (Biorad).

195

196 *Gene expression analysis*

197 For the analysis of all data, the reference gene chosen was luciferase. The relative amount of
198 expression of the target genes was determined by the $\Delta\Delta$ CT method. The efficiency of PCR, as
199 measured by the slope of a standard curve using serial cDNA dilutions, was between 1.85 and
200 2.

201

202 *Statistical analysis*

203 Results are expressed as mean \pm SEM and analyzed by one-way ANOVA supplemented by a
204 Tukey test with the GraphPad Prism software. Pearson correlation tests, principal component
205 analysis (PCA) and qPCR results were performed with the software R (Rcmdr, FactominR).
206 Differences were considered statistically significant at $P\leq 0.05$.

207

208 **RESULTS**

209

210 *Tissue weights and plasma metabolite levels*

211 In order to better understand the mechanisms underlying the development of fatty liver in
212 overfed mule duck, zootechnical data (tissue weights) and plasma data were analysed during
213 overfeeding (n=32 to each sampling points: M4, M12 and M22) (Table 2). A significant
214 increase in liver ($P<0.0001$), abdominal fat ($P<0.0001$) and subcutaneous adipose tissue weight

215 (P<0.0001) was observed during all the overfeeding period while muscle weight increased
216 significantly at the beginning of the overfeeding (between meal 4 and meal 12) and then
217 remained stable until the end (P=0.0007).

218 In addition, analysis of plasma parameters showed a significant increase of glucose (P=0.0052),
219 triglycerides (P<0.0001) and total cholesterol (P<0.0001) levels during overfeeding (Table 2).
220 Interesting results were obtained by conducting a PCA comparing zootechnical parameters to
221 plasma data (Fig. 1A). A significant correlation appears between tissue weights (liver,
222 abdominal fat and subcutaneous adipose tissue) and total cholesterol levels with a greater
223 significant correlation between liver weights and total cholesterol levels during overfeeding
224 (r=0.88, P<0.0001) (Fig. 1B). Pearson correlation tests showed that the correlation between
225 liver weight and cholesterolemia is only marked at the end of overfeeding (r=-0.17, ns, r=0.24,
226 ns and r=0.58, P<0.001 for M4, M12 and M22 respectively). These early results, which suggest
227 a significant impact of overfeeding in cholesterol metabolism in mule ducks, and mainly at the
228 end of the overfeeding period, will be supported by the following cholesterol metabolism gene
229 expression study.

230

231 ***Gene expression and western blot analysis***

232

233 *Glucose metabolism*

234 As concerned genes involved in glucose metabolism, in liver (Table 3), we observed a
235 significant increase of the expression of Acetyl CoA Carboxylase (*acox*), Succinate
236 Dehydrogenase Complex - Subunit A (*sdha*), Glucose Transporters (*glut1/2/8*), Insulin
237 Receptor (*insr*), Insulin like Growth Factor 1(*igf1*) and Carbohydrate Response Element
238 Binding Protein (*chrebp*) during all the overfeeding period (P<0.0001). For genes Hexokinase
239 1 (*hk1*), Glyceraldehyde 3 Phosphate Dehydrogenase (*gapdh*) and *glut3*, their expression
240 significantly increase on the second period of overfeeding (between M12 and M22) (P<0.0001).
241 For Enolase Alpha (*eno1*), we observed a significant increase between M4 and M22
242 (P=0.0078).

243 In muscle (Table 3), the expression of *gapdh* increased significantly at the beginning of the
244 overfeeding between M4 and M12 (P=0.003) and then stabilized, while the expression of *acox*
245 and *glut8* significantly increased between M12 and M22 (P=0.0004 and P<0.0001
246 respectively). For *sdha* and *glut1*, we observed significant increase between M4 and M22
247 (P=0.0273 and P=0.0021 respectively).

248 In abdominal fat (Table 3), only the expression of *sdha*, *insr* and *chrebp* showed a significant
249 increase between M4 and M12 then a stabilization (P=0.0003, P=0.0086 and P=0.018
250 respectively).

251

252 *Lipid metabolism*

253 In liver (Table 4), the expression of Acyl CoA Synthetase Long-chain 1 (*acs1l*) and Stéaryl
254 CoA désaturase (*scd1*) significantly increased during all the overfeeding (P<0.0001). Others
255 genes, also involved in *de novo* lipogenesis, such as Fatty Acid Synthase (*fas*) and Diglyceride
256 Acyl Transferase 2 (*dgat2*), had an expression that increases significantly between M4 and M12
257 and then stabilized until the end of the overfeeding (P=0.0011 and P<0.0001 respectively).

258 Concerning genes involved in β -oxidation, Acyl CoA Dehydrogenase (*acad*) and Hydroxyacyl-
259 CoA Dehydrogenase (*hadh*) evolved in the same way with a significant increase during the
260 overfeeding (P<0.0001) while Carnitine Palmitoyl Transferase A (*cpt1a*) only increased at the
261 end of the overfeeding between M12 and M22 (P<0.0001).

262 For genes implicated in lipoprotein formation and transport, we observed a significant increase
263 of their expression from the beginning of the overfeeding (between M4 and M12). While
264 Apolipoprotein A (*apoa*), Glycerol-3-Phosphate Acyltransferase 1 (*gpac1*) (P<0.0001) and
265 Microsomal Triglycerides Transfer Protein (*mttp*) stabilized (P=0.0017), we can observe a
266 continuous increase for Apolipoprotein B (*apob*), Carnitine Palmitoyl Transferase A (*cept1*),
267 Perilipin 2 (*plin2*), Lipase Maturation Factor 1 (*lmf1*); and Fatty Acid Translocase/Cluster of
268 Differentiation 36 (*fat/cd36*) expressions until the end of the overfeeding (P<0.0001) and an
269 overexpression of Fatty Acid Binding Protein 4 (*fabp4*) only at the end of the overfeeding
270 period (between M12 and M22) (P<0.0001). For the lipoprotein receptors Low Density
271 Lipoprotein Receptor (*ldlr*) and Very Low Density Lipoprotein Receptor (*vldlr*), we have an
272 identical variation of their expression with a significant increase during all the overfeeding
273 (P<0.0001). For genes implicated in cholesterol synthesis, we observed an increase of the
274 expression of Hydroxymethylglutaryl-CoA Reductase (*hmgcr*) at the end of the overfeeding
275 (between M12 and M22) (P=0.0004), a significant increase of Sterol O-acyltransferase 1
276 (*soat1*) during all the overfeeding and an increase of the expression of Cytochrome P450 family
277 5 (*cyp51a*) only in the middle of the overfeeding period (M12) (P=0.0279). To finish, the
278 expression of the transcription factors associated with these previous genes, Peroxisome
279 Proliferator Activated Receptor Alpha (*ppara*), Gamma (*ppar γ*) and Liver X Receptor Alpha
280 (*lxra*) significantly increased all during the overfeeding (P<0.0001).

281 In muscle (Table 5), as genes involved in lipogenesis *de novo*, only the expression of ATP
282 Citrate Lyase (*acly*) and *acs11* increased; between M12 and M22 for *acly* (P=0.0285) and
283 between M4 and M12 for *acs11* (P<0.0001). The expression of β -oxidation genes *acad* and *hadh*
284 increased significantly all during the overfeeding (P=0.0121 and P=0.0001 respectively)
285 whereas *cyp51a* expression only increased at the end of the overfeeding (between M12 and
286 M22) (P<0.0001). For genes involved in lipoprotein formation and transport, the expression of
287 *cept1* and *fat/cd36* increased on the second period of overfeeding (between M12 and M22)
288 (P=0.0342 and P=0.0007 respectively). For *gpat1* and *lmf1*, the expression were significantly
289 different between M4 and M22 (P=0,021 and P<0.0001 respectively).

290 In abdominal fat (Table 5), we were not able to detect as much gene expressions as in the liver
291 or in muscle. We only observed a significant increase of the expression of *acs11* during
292 overfeeding between M4 and M22 (P=0.0011). *apoa* presents a slight increase between M4 and
293 M12 then return to its basal level (P=0,014).

294

295 *mTOR pathway*

296 Because we observed significant modifications in insulin and glucose metabolisms mainly in
297 liver, we decided to investigate the protein kinase B (Akt)/ target of rapamycin (TOR) signalling
298 pathway during overfeeding only in liver of mule ducks by western blot analysis. As illustrated
299 in Fig. 2, we observed a significant increase of phosphorylation of Akt on Ser 473 (P=0.0117)
300 between M4 and M22 and an increase of phosphorylation of S6 on Ser 235/236 (P<0.0001) at
301 the end of the overfeeding (between M12 and M22).

302 Gene expression analysis of *akt*, *mtor* and ribosomal protein S6 kinase (*s6k1*) in the liver
303 showed a significant increase of their expression during all the overfeeding (P<0.0001) (Table
304 5). In muscle, only *s6k1* expression significantly increased during the overfeeding (P=0.0161).
305 No gene expression variation was observed in abdominal fat.

306

307 *Cellular stress*

308 Regarding to gene expression analysis in liver (Table 6), the expression of Activating
309 Transcription Factor 4 (*atf4*) and Asparagine Synthetase (*asns*), involved in the endoplasmic
310 reticulum (ER) stress and also in a lesser extent in amino acid deficiency pathway, significantly
311 increased during all the overfeeding period (P<0.0001). Similarly, the expression of genes
312 involved in macroautophagy [Autophagy related gene 4b/8/9 (*atg4b/8/9*), Sequestosome 1
313 (*sqstm1*)], in chaperone-mediated autophagy (CMA) [Lysosome-associated membrane protein
314 2 (*lamp2a*)], in apoptosis [Caspase 3/8/9 (*cas3/8/9*)] and in global cellular stress [Heat Shock

315 Protein beta 1 (*hsbp1*) significantly increased during all the overfeeding period ($P < 0.0001$).
316 Hepatic steatosis would therefore induce ER stress, which would then activate the expression
317 of genes of eIF2 α /ATF4 and autophagy pathways.

318 In muscle (Table 6), *asns* increased only at the end of the overfeeding between M12 and M22
319 ($P = 0.001$). *atg4b*, *hsbp1* and *casp8* expressions increased significantly between M4 and M22
320 ($P = 0.0157$; $P = 0.0089$; $P = 0.0006$ respectively). *Casp9* increased significantly only between
321 M12 and M22 ($P = 0.0007$) while *casp3* increased between M4 and M12 and then stabilized
322 ($P = 0.0002$).

323 In abdominal fat (Table 6), only *casp8* seems to be impacted by the overfeeding with a
324 significant increase of its expression during all the overfeeding ($P = 0.0113$).

325

326 ***Melting rate and mRNA expression of FABP4***

327

328 Pearson correlation analysis conducted on mRNA expression of *fabp4* and melting rate
329 achieved on livers of mule ducks at the end of overfeeding (M22) showed a significant negative
330 correlation ($r = -0.67$, $P < 0.05$) (Fig. 3). No significant correlations were observed between
331 melting rate and others genes overexpressed at the end of overfeeding.

332

333 **DISCUSSION**

334

335 The production of “foie gras” is subjected to numerous economic and regulatory constraints as
336 well as ethics questions. To respond to these, rearing and overfeeding conditions must be
337 optimized. For this purpose, different options are available: identifying markers of the
338 development of hepatic steatosis, but also better understand the mechanisms underlying its
339 development, in order to optimize it. In this experiment, the impact of the overfeeding on
340 intermediate metabolism and cellular stress was studied during hepatic steatosis establishment
341 in mule duck. This kinetic study was carried out during all the overfeeding period with three
342 points of sampling: at the beginning (4th meal), at the middle (12th meal) and at the end (22nd
343 meal). In general, studies that have been conducted on hepatic steatosis in ducks focus more on
344 lipid metabolism (6, 19, 48) or glucose metabolism (48) with a comparison between different
345 duck’s genotype or between overfed and non-overfed ducks (18, 19, 49). Our study explored
346 gene expression on the entire duration of the overfeeding period and also provided additional
347 information on intermediate metabolism, insulin and mTOR pathway, cholesterol metabolism
348 and global cellular stress potentially activated during the development of overfeeding.

349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381

Overfeeding resulted in continuous weight gain of liver, abdominal fat and subcutaneous adipose tissue. By contrast, muscle weight gain is marked at the beginning of the overfeeding then stabilizes. The increase of tissue weights during the overfeeding is accompanied by an increase of triglyceridemia and total cholesterol, as already observed in previous studies (1, 12, 48). But interestingly, this study has revealed a significant positive correlation between liver weight and plasma total cholesterol (Fig. 1) supporting the idea that cholesterol metabolism could be impacted during overfeeding in mule ducks and may be used as potential biomarker of hepatic steatosis development. To better understand the involvement of the cholesterol metabolism on hepatic steatosis development, we analysed the expression of genes involved in cholesterol synthesis (*hmgcr*, *cyp51a*) and esterification (*soat1*). The expression of *hmgcr*, involved in the mevalonate pathway that produces cholesterol precursors, increases during the second half of the overfeeding period, in agreement with data observed in NAFLD patients (34). A significant increase of *cyp51a*, responsible of another step of the cholesterol biosynthetic pathway, was also observed during the overfeeding period suggesting that the whole cholesterol biosynthetic pathway is probably activated during the development of the hepatic steatosis induced by overfeeding in mule ducks. Furthermore, the significant increase of *soat1* (also called *acat*) expression during all the overfeeding period suggests the conversion of cholesterol to its storage form, cholesteryl esters (38), probably in response to the accumulation of free cholesterol as previously indicated by Rogers *et al.* (38). A similar situation is also observed in human with NAFLD, where free cholesterol content increases in the liver and the induction of *acat* activity results in the esterification of excess synthesized cholesterol (46). *apob* and *mtfp* known to be involved in triglyceride transport from the liver to peripheral tissues were also overexpressed in liver of mule ducks during the development of the steatosis. A correlation analysis performed between liver weights and mRNAs expression of genes linked to cholesterol metabolism revealed a significant and positive correlation for *apob* and *soat1* ($r=0.6906$ $P<0.0001$ and $r=0.6696$ $P<0.0001$ respectively). Supported by significant correlations between the expression of key genes involved in cholesterol metabolism and liver weight, the present study indicated that the establishment of hepatic steatosis during overfeeding in mule ducks seems to be closely related to a strong modification of cholesterol metabolism based on the induction of cholesterol synthesis and the storage of free cholesterol as cholesterol esters.

382 Overfeeding in duck also deeply modifies glucose metabolism. It first affects glucose transport,
383 as the hepatic expression of *glut1/2/8* continuously increases during overfeeding while that of
384 *glut3* seems to be rather activated during the second half of the overfeeding period. On the
385 contrary, no expression of *glut2* was detected in muscle or abdominal fat confirming previous
386 results obtained by Tavernier *et al.* (48) in ducks and Kono *et al.* (28) in chickens. Consistently,
387 the hepatic expression of *chrebp*, the transcription factor activated by glucose and involved in
388 the setting up of glucose transporters (24, 25, 50), increases during overfeeding. Therefore,
389 altogether, these results suggest that the liver of mule ducks responds to overfeeding by
390 increasing its glucose uptake capacity.

391
392 *Hkl* and *enol* that catalyse the first and the last step of the glycolysis reaction, respectively, and
393 *sdha* that helps to produce energy through its implication in the respiratory chain (Krebs cycle)
394 (18) are all overexpressed in the liver of mule ducks during overfeeding. During the same time,
395 a small increase of plasma glucose level occurs but only at the end of the overfeeding period
396 suggesting that, mule duck is able to tackle the dietary carbohydrate overload by stimulating
397 glucose uptake and glycolysis. This response seems to be mainly restricted to the liver as
398 relatively small induction of carbohydrate metabolism occurs in muscle and adipose tissue
399 during overfeeding. However, despite a strong induction of the expression of genes involved in
400 glucose metabolism, the final slight increase in blood glucose occurring at the end of the
401 overfeeding period may suggest that the metabolism has reached a limit in the utilization of
402 glucose.

403
404 During overfeeding in duck, the fate of glucose is to be converted into lipids in the liver. A
405 significant increase in the expression of *acox*, a *chrebp* target gene involved in the first step of
406 lipogenesis, occurs in liver, probably stimulated by insulin secretion as observed in mammals
407 (36, 42). Expression of *scd1*, *acs11* and the transcription factors *ppary* and *lxra* rises during the
408 overfeeding period, whereas the expression of *fas* and *dgat2* reach their highest level during the
409 first half of overfeeding period. As previously demonstrated, these genes involved in *de novo*
410 lipogenesis enhance during overfeeding and lead to the development of an important hepatic
411 steatosis in mule ducks (31, 40, 48). The genes encoding *fas*, *scd1* and *dgat2* were previously
412 observed as overexpressed in the liver of overfed Pekin and Muscovy ducks, 12 hours after the
413 last overfeeding meal (19) and *acs11* is known to be up-regulated in liver of overfed geese (57).
414 Therefore, the present study confirms previous findings proposing *dgat2* as a key enzyme
415 responsible for the accumulation of lipids in the liver of overfed mule ducks (48, 49).

416 Even if *acly* expression does not evolve in the liver during overfeeding in our study, it increases
417 in the muscle in the second part of overfeeding period. The role of this enzyme is to convert
418 citrate to acetyl-CoA in the cytosol allowing glucose to serve as substrate for *de novo*
419 lipogenesis. A study on 3 days old overfed chicks demonstrated a significant increase in *acly*
420 expression in the liver (45). Moreover, Hérault *et al.* showed a positive correlation between
421 liver weight and *acly* mRNAs expression during overfeeding in Pekin and muscovy ducks (19).
422 This kind of regulation was not recorded in the present study. Nevertheless, an increase in the
423 expression of *acly* may have occur at the early beginning of overfeeding, between the first and
424 the fourth meal and remain stable during the whole overfeeding period thus explaining the
425 discrepancy with previous demonstrations. Finally, our results indicate that during overfeeding
426 of mule ducks, dietary carbohydrates are up taken and converted into lipids, mainly by the liver,
427 and exported to peripheral tissues. The adipose tissues (in priority) and probably the muscle (at
428 a lower level) constitute places of storage of the lipids as shown by the evolution of the weights
429 of these tissues during overfeeding.

430

431 Insulin is considered as a key regulator of glucose and lipid metabolism. In the present
432 experiment, as glucose metabolism modulations were preferentially observed in the liver, we
433 therefore analysed the activation of two proteins involved in the insulin and amino acids cell
434 signalling pathway only in the liver. We found that phosphorylation of Akt and S6 increases
435 during the overfeeding period. Several studies have shown that most patients with NASH
436 exhibit insulin resistance (7, 43) but our results seem to indicate that no insulin resistance occurs
437 during the development of hepatic steatosis in overfed mule ducks because Akt/mTOR
438 signalling is not inhibited. These observations are concomitant with the increase of *akt*, *mtor*
439 and *s6kl* expressions in the liver all along the overfeeding period. A significant increase in *insr*
440 expression also occurs throughout overfeeding in the liver and during the first half of
441 overfeeding period in the abdominal fat tissue. *Igfl* expression significantly increase in liver
442 throughout overfeeding and only at the end of overfeeding in muscle. Altogether, these results
443 suggest that, in order to cope with a very important intake of carbohydrates, mule duck increases
444 the number of hepatic insulin receptors and boost insulin sensitivity to raise glucose uptake and
445 use it *via* glycolysis and lipogenesis.

446

447 Beta-oxidation is a predominantly mitochondrial pathway for the degradation of fatty acids. It
448 is carried out under the action of *cpt1a*, enabling the transport of acyl-coA into the
449 mitochondria, and several enzymes including *acad* and *hadh* involved in dehydrogenation of

450 acyl-coA and the production of β -cetoacyl-CoA, respectively. In the liver of mule ducks, the
451 expression of *cpt1a* increases significantly at the end of the overfeeding, whereas that of *acad*
452 and *hadh* rises during the first half of overfeeding, coupled with a significant overexpression of
453 the *ppara* transcription factor throughout the overfeeding period. In muscle, *cpt1a*, *acad* and
454 *hadh* expressions also increase at the end of overfeeding. These results suggest an induction of
455 beta-oxidation in liver and muscle with a more pronounced effect in the liver compared to
456 muscle. Thus, during overfeeding, a concomitant increase in lipogenesis and beta-oxidation is
457 set up. The concomitant strong synthesis of lipids and use of lipids as energy substrate suggest
458 that overfed mule ducks may be trying to limit the accumulation of lipids. Our results are also
459 consistent with previous works (48) showing that plasma free fatty acids early increased in
460 overfed mule ducks and remained at high level during all the duration of the overfeeding period.
461 Free fatty acids such as palmitic acid has emerged as lipotoxic agents affecting cell signaling
462 cascades and death receptors, endoplasmic reticulum stress, mitochondrial function, and
463 oxidative stress (33). Therefore, the induction of beta-oxidation by overfeeding in mule ducks
464 would allow removing part of newly synthesized lipids and thus avoid lipotoxicity.

465
466 Concerning genes involved in lipoprotein formation (*apoa*, *apob*, *mttp*), lipid transport (*fabp4*,
467 *fat/cd36*) and receptors (*vldlr*, *ldlr*), we observed an overexpression of all of these genes during
468 overfeeding mostly in liver and very little or not at all in muscle. The high hepatic lipid re-
469 uptake associated with the overexpression of *ldlr*, *vldlr*, *fat/cd36* and *fabp4*, is well-known in
470 mule duck (48) and is confirmed in the present study. These results suggest that during
471 overfeeding newly synthesized lipids are exported by the liver to peripheral tissues, probably
472 to reduce lipotoxicity. However, the lack in lipids uptake at peripheral level leads to a return of
473 the lipids to the liver, which re-uptake them via fatty acid transporters or lipoprotein receptors,
474 leading to a greater lipid accumulation in the liver at the end of the overfeeding. Among these
475 various transporters, we notice the huge overexpression of *fabp4* in the liver at the end of the
476 overfeeding period. Surprisingly, we observed a significant negative correlation between *fabp4*
477 mRNAs expression and the melting rate achieved at the end of the overfeeding. Numerous
478 studies suggest that fatty acid composition may be controlled by genes related to lipid synthesis
479 and fatty acid metabolism. Studies performed on cattle demonstrated the relation between the
480 composition of intramuscular fatty acid and genes involved in lipid metabolism such as *fabp4*
481 that contribute to fatty acid deposition (5, 23). In order to find genetic markers associated with
482 fatty acid composition in beef, Hoashi *et al.* (23) found an effect of the polymorphisms of *fabp4*
483 on the fatty acid composition of carcasses making *fabp4*, a promising candidate for beef quality

484 biomarker (flavour and tenderness). Blecha *et al.* (5) suggested too that *fabp4* may participate
485 in the regulation of intramuscular fatty acid metabolism in yaks and could be used as markers
486 to improve yak meat quality. In general, polyunsaturated fatty acid levels are positively
487 correlated with *fabp4*. In our study, we can hypothesize that the hepatic fatty acids resulting
488 from *fabp4* reuptake could be of different nature, ie lipids with a higher melting point and
489 therefore less mobile during cooking.

490

491 Our results strongly show that mule ducks respond to overfeeding by significant modifications
492 of their intermediate metabolism including activation of the insulin pathway and induction of
493 beta-oxidation. However, what about cellular defence mechanisms put in place by ducks to
494 overcome this state of steatosis induced by overfeeding. What are the necessary mechanisms
495 induced to maintain this steatosis without switching to a pathological state such as fibrosis? To
496 try to answer some of these questions, we analyzed the expression of several genes linked to
497 cellular stress pathways.

498 One of these pathways was autophagy that contributes to maintain cellular homeostasis.
499 Activated under conditions of nutrient deficiency or deprivation, or cellular stress (35),
500 autophagy allows the degradation of part of the cytoplasm including damaged proteins,
501 organelles and lipids, leading to the production of amino acids and other nutrients that could be
502 recycled for the synthesis of macromolecules or used as a source of energy (15, 17).
503 Interestingly, autophagy is known to be regulated by mTOR pathway *via* ATG1/ULK
504 phosphorylation (26) but is also mostly linked to ER stress (56). In mammalian models, the
505 inhibition of the autophagic process and more especially the inhibition of lipophagy results in
506 triglyceride accumulation into the liver (44). Consequently, it seemed relevant to study
507 autophagy-related gene expression in mule ducks during overfeeding. We particularly focused
508 our attention on *atg8 (lc3)* and *atg9*, two proteins known to be associated with the number of
509 autophagosomes formed in yeast (58); *sqstm1 (p62)*, an autophagy receptor that links
510 ubiquitinated proteins to LC3; and *atg4b*, which hydrolyses the bond between *atg8* and
511 phosphatidylethanolamine (PE), making it a specific marker for this organelle (8). Surprisingly,
512 the present study reveals a significant upregulation of the expression of the genes related to
513 autophagy during overfeeding. On the contrary, the induction of the expression of the
514 cytochrome P450 2E1 (*cyp2e1*) observed in our study suggests that autophagy is suppressed by
515 overfeeding of ducks. Indeed, *cyp2e1* has been shown to mediate the upregulation of oxidative
516 stress-suppressed autophagy, thus leading to lipid accumulation in cultured liver cells (52). In

517 mice, it has been proposed that high-fat diets, by reducing LAMP2A, could inhibit the
518 degradation of PLIN2 by the chaperone-mediated autophagy leading to the reduction of
519 lipophagy (54). However, the expression of both *lamp2a* and *plin2* enhanced during the
520 development of the hepatic steatosis in duck conflicting with the idea of an inhibition of
521 lipophagy promoting triglyceride accumulation into the liver of mule ducks during overfeeding.
522 Therefore, if autophagy is effectively inhibited in overfed mule ducks, it must be strictly
523 demonstrated using autophagic flux analyses.

524 Another cellular stress pathway is eIF2 α /ATF4. Activated under amino acid deprivation, the
525 eIF2 α /ATF4 pathway is before all a target of transmembrane protein PKR-related Endoplasmic
526 Reticulum Kinase (PERK) activated in ER stress (2, 16, 27, 39). EIF2 α /ATF4 pathway have
527 the ability to triggers the transcriptional expression of genes involved in amino acid metabolism
528 or resistance to oxidative stress. Throughout the overfeeding period, a significant increase of
529 the expression of *atf4* and its target *asns* was observed in the liver of mule ducks and to a lower
530 level, in the muscle. This strong hepatic induction of *atf4* and *asns* probably reflects the strong
531 nutritional imbalance of the feed used during overfeeding. Mainly composed of corn, this diet
532 exhibits a very low content in protein and amino acid that probably doesn't cover amino acid
533 mule duck requirement.

534

535 At the end of the overfeeding period, overexpression of *casp3*, 8 and 9 involved in apoptosis
536 enhanced in liver and in muscle. At the same time, the expression of the protein chaperone
537 *hsbp1*, involved in stress resistance and known to reduce oxidative stress and suppress some
538 modes of apoptosis or cell death (51) increased. Altogether, these results indicated that
539 mechanisms are implemented during overfeeding to limit cellular stress and apoptosis during
540 the development of the hepatic steatosis.

541

542 CONCLUSION

543

544 To conclude, the present study contributes to a better understanding of the physiological
545 mechanisms triggered during overfeeding in the mule duck and underlying to the development
546 of hepatic steatosis. In order to cope with an overload of dietary carbohydrates, mule ducks
547 seem to adapt its metabolism by first increasing its capacity of glucose uptake and
548 transformation of glucose into lipids. These mechanisms are probably enabled by an increasing
549 activation of the insulin-signaling pathway throughout the overfeeding period. In addition, a

550 strong lipoprotein synthesis mainly occurs in the liver but exported lipids are reuptake by the
551 liver at the end of the overfeeding period, which strongly contributes to the development of the
552 hepatic steatosis. Nevertheless, beta-oxidation is simultaneously stimulated probably to limit a
553 too important accumulation of lipids and avoid lipotoxicity of free fatty acids. During
554 overfeeding, mule ducks seem to accordingly adapt tissue response to resist to the setup various
555 cellular stress including autophagy, amino acid deficiency, ER and oxidative stress or apoptosis.
556 Altogether, these mechanisms enables mule ducks to efficiently handle this huge starch
557 overload while keeping the liver in a state of steatosis without switching to a pathological
558 condition.

559 This study also bring to light potential biomarker candidates of hepatic steatosis as plasma
560 cholesterol for liver weight. The development of robust non-invasive biomarker of liver weight
561 and melting rate could be of great interest to monitor in real-time the development of hepatic
562 steatosis of mule duck during overfeeding and predict the quality of the product.

563

564 ACKNOWLEDGMENTS

565

566 We thank the “Conseil Départemental des Landes (CD40)” and Nutricia from the cooperative
567 group Maisadour for financing this work. We also thank the technical staff of INRA Artiguères
568 for rearing ducks. We are grateful to Emilie Bonin for performing Fluidigm analysis [Génopole
569 Toulouse/Midi-Pyrénées, Plateau Transcriptomique Quantitative (TQ), Toulouse, France].

570

571 REFERENCES

572

- 573 1. **Baéza E, Rideau N, Chartrin P, Davail S, Hoo-Paris R, Mourot J, Guy G,**
574 **Bernadet MD, Juin H, Meteau K.** Canards de Barbarie, Pékin et leurs hybrides: aptitude à
575 l’engraissement. *INRA Prod Anim* 18: 131–141, 2005.
- 576 2. **Barbosa-Tessmann IP.** Activation of the Human Asparagine Synthetase Gene by the
577 Amino Acid Response and the Endoplasmic Reticulum Stress Response Pathways Occurs by
578 Common Genomic Elements. *J. Biol. Chem.* (June 15, 2000). doi: 10.1074/jbc.M000004200.
- 579 3. **Barthel A, Schmoll D.** Novel concepts in insulin regulation of hepatic
580 gluconeogenesis. *Am J Physiol-Endocrinol Metab* 285: E685–E692, 2003.
- 581 4. **Bénard G, Bénard P, Prehn D, Bengone T, Jouglar JY, Durand S.** Démonstration
582 de la réversibilité de la stéatose hépatique obtenue par gavage de canards mulards. *Etude*
583 *Réalis Sur* 3: 49–52, 1998.
- 584 5. **Blecha IMZ, Siqueira F, Ferreira ABR, Feijó GLD, Torres Júnior RAA,**
585 **Medeiros SR, Sousa II, Santiago GG, Ferraz ALJ.** Identification and evaluation of
586 polymorphisms in FABP3 and FABP4 in beef cattle. *Genet Mol Res* 14: 16353–16363, 2015.

- 587 6. **Chartrin P, Bernadet M-D, Guy G, Mourot J, Hocquette J-F, Rideau N, Duclos**
588 **MJ, Baéza E.** Does overfeeding enhance genotype effects on liver ability for lipogenesis and
589 lipid secretion in ducks? *Comp Biochem Physiol A Mol Integr Physiol* 145: 390–396, 2006.
- 590 7. **Chitturi S, Abeygunasekera S, Farrell GC, Holmes-Walker J, Hui JM, Fung C,**
591 **Karim R, Lin R, Samarasinghe D, Liddle C, Weltman M, George J.** NASH and insulin
592 resistance: Insulin hypersecretion and specific association with the insulin resistance
593 syndrome. *Hepatology* 35: 373–379, 2002.
- 594 8. **Codogno P, Meijer AJ.** Autophagy and signaling: their role in cell survival and cell
595 death. *Cell Death Differ* 12: 1509–1518, 2005.
- 596 9. **Desvignes T, Fauvel C, Bobe J.** The nme gene family in zebrafish oogenesis and
597 early development. *Naunyn Schmiedebergs Arch Pharmacol* 384: 439–449, 2011.
- 598 10. **Fantini J, Epand RM, Barrantes FJ.** Cholesterol-Recognition Motifs in Membrane
599 Proteins. In: *Direct Mechanisms in Cholesterol Modulation of Protein Function*, edited by
600 Rosenhouse-Dantsker A, Bukiya AN. Springer International Publishing, p. 3–25.
- 601 11. **Fernandez X, Bouillier-Oudot M, Molette C, Bernadet MD, Manse H.** Duration of
602 transport and holding in lairage at constant postprandial delay to slaughter--Effects on fatty
603 liver and breast muscle quality in mule ducks. *Poult Sci* 90: 2360–2369, 2011.
- 604 12. **Gontier K, André J-M, Bernadet M-D, Ricaud K, Davail S.** Insulin effect on
605 lipogenesis and fat distribution in three genotypes of ducks during overfeeding. *Comp*
606 *Biochem Physiol A Mol Integr Physiol* 164: 499–505, 2013.
- 607 13. **González-Rodríguez á, Mayoral R, Agra N, Valdecantos MP, Pardo V,**
608 **Miquilena-Colina ME, Vargas-Castrillón J, Lo Iacono O, Corazzari M, Fimia GM,**
609 **Piacentini M, Muntané J, Boscá L, García-Monzón C, Martín-Sanz P, Valverde á M.**
610 Impaired autophagic flux is associated with increased endoplasmic reticulum stress during the
611 development of NAFLD. *Cell Death Dis* 5: e1179–e1179, 2014.
- 612 14. **Guy G, Rousselot-Pailley D, Gourichon D.** Comparaison des performances de l'oie,
613 du canard mulard et du canard de Barbarie soumis au gavage. In: *Annales de zootechnie*.
614 1995, p. 297–305.
- 615 15. **Ha J, Guan K-L, Kim J.** AMPK and autophagy in glucose/glycogen metabolism.
616 *Mol Aspects Med* 46: 46–62, 2015.
- 617 16. **Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calton M, Sadri N, Yun C,**
618 **Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D.** An Integrated
619 Stress Response Regulates Amino Acid Metabolism and Resistance to Oxidative Stress. *Mol*
620 *Cell* 11: 619–633, 2003.
- 621 17. **He C, Klionsky DJ.** Regulation Mechanisms and Signaling Pathways of Autophagy.
622 *Annu Rev Genet* 43: 67–93, 2009.
- 623 18. **Hérault F, Duby C, Baéza E, Diot C.** Adipogenic genes expression in relation to
624 hepatic steatosis in the liver of two duck species. *animal* 12: 2571–2577, 2018.
- 625 19. **Hérault F, Saez G, Robert E, Mohammad AA, Davail S, Chartrin P, Baéza E,**
626 **Diot C.** Liver gene expression in relation to hepatic steatosis and lipid secretion in two duck
627 species. *Anim Genet* 41: 12–20, 2009.
- 628 20. **Hermier D, Guy G, Guillaumin S, Davail S, André J-M, Hoo-Paris R.** Differential
629 channelling of liver lipids in relation to susceptibility to hepatic steatosis in two species of
630 ducks. *Comp Biochem Physiol B Biochem Mol Biol* 135: 663–675, 2003.
- 631 21. **Hermier D, Saadoun A, Salichon M-R, Sellier N, Rousselot-Paillet D, Chapman**
632 **MJ.** Plasma lipoproteins and liver lipids in two breeds of geese with different susceptibility to
633 hepatic steatosis: Changes induced by development and force-feeding. *Lipids* 26: 331–339,
634 1991.

- 635 22. **Hermier D, Salichon MR, Guy G, Peresson R.** Differential channelling of liver
636 lipids in relation to susceptibility to hepatic steatosis in the goose. *Poult Sci* 78: 1398–1406,
637 1999.
- 638 23. **Hoashi S, Hinenoya T, Tanaka A, Ohsaki H, Sasazaki S, Taniguchi M, Oyama K,**
639 **Mukai F, Mannen H.** Association between fatty acid compositions and genotypes of FABP4
640 and LXR-alpha in Japanese Black cattle. *BMC Genet* 9: 84, 2008.
- 641 24. **Iizuka K, Bruick RK, Liang G, Horton JD, Uyeda K.** Deficiency of carbohydrate
642 response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis. *Proc*
643 *Natl Acad Sci* 101: 7281–7286, 2004.
- 644 25. **Ishii S, Iizuka K, Miller BC, Uyeda K.** Carbohydrate response element binding
645 protein directly promotes lipogenic enzyme gene transcription. *Proc Natl Acad Sci* 101:
646 15597–15602, 2004.
- 647 26. **Jung CH, Ro S-H, Cao J, Otto NM, Kim D-H.** mTOR regulation of autophagy.
648 *FEBS Lett* 584: 1287–1295, 2010.
- 649 27. **Kilberg MS, Balasubramanian M, Fu L, Shan J.** The Transcription Factor Network
650 Associated With the Amino Acid Response in Mammalian Cells. *Adv Nutr* 3: 295–306, 2012.
- 651 28. **Kono T, Nishida M, Nishiki Y, Seki Y, Sato K, Dr YA.** Characterisation of glucose
652 transporter (GLUT) gene expression in broiler chickens. *Br Poult Sci* 46: 510–515, 2005.
- 653 29. **Lansard M, Panserat S, Plagnes-Juan E, Seiliez I, Skiba-Cassy S.** Integration of
654 insulin and amino acid signals that regulate hepatic metabolism-related gene expression in
655 rainbow trout: role of TOR. *Amino Acids* 39: 801–810, 2010.
- 656 30. **Latil G, Auvergne A, Babilé R.** Consommation du canard mulard en gavage. *Relat.*
657 *Avec Perform. Zootech. Qual. Foie Gras.* .
- 658 31. **Leveille GA, Romsos DR, Yeh Y-Y, O’Hea EK.** Lipid Biosynthesis in the Chick. A
659 Consideration of Site of Synthesis, Influence of Diet and Possible Regulatory Mechanisms.
660 *Poult Sci* 54: 1075–1093, 1975.
- 661 32. **Marandel L, Labbe C, Bobe J, Le Bail P-Y.** nanog 5'-upstream sequence, DNA
662 methylation, and expression in gametes and early embryo reveal striking differences between
663 teleosts and mammals. *Gene* 492: 130–137, 2012.
- 664 33. **Marra F, Svegliati-Baroni G.** Lipotoxicity and the gut-liver axis in NASH
665 pathogenesis. *J Hepatol* 68: 280–295, 2018.
- 666 34. **Min H-K, Kapoor A, Fuchs M, Mirshahi F, Zhou H, Maher J, Kellum J,**
667 **Warnick R, Contos MJ, Sanyal AJ.** Increased Hepatic Synthesis and Dysregulation of
668 Cholesterol Metabolism Is Associated with the Severity of Nonalcoholic Fatty Liver Disease.
669 *Cell Metab* 15: 665–674, 2012.
- 670 35. **Mizushima N, Komatsu M.** Autophagy: Renovation of Cells and Tissues. *Cell* 147:
671 728–741, 2011.
- 672 36. **Niemeyer H, Ureta T, Clark-Turri L.** Adaptive character of liver glucokinase. *Mol*
673 *Cell Biochem* 6: 109–126, 1975.
- 674 37. **Pilo B, George JC.** Diurnal and seasonal variation in liver glycogen and fat in relation
675 to metabolic status of liver and m. pectoralis in the migratory starling, *Sturnus roseus*,
676 wintering in India. *Comp Biochem Physiol A* 74: 601–604, 1983.
- 677 38. **Rogers MA, Liu J, Song B-L, Li B-L, Chang CCY, Chang T-Y.** Acyl-
678 CoA:cholesterol acyltransferases (ACATs/SOATs): Enzymes with multiple sterols as
679 substrates and as activators. *J Steroid Biochem Mol Biol* 151: 102–107, 2015.
- 680 39. **Roybal CN, Hunsaker LA, Barbash O, Jagt DLV, Abcouwer SF.** The Oxidative
681 Stressor Arsenite Activates Vascular Endothelial Growth Factor mRNA Transcription by an
682 ATF4-dependent Mechanism. *J Biol Chem* 280: 20331–20339, 2005.
- 683 40. **Saadoun A, Leclercq B.** In Vivo Lipogenesis of Genetically Lean and Fat Chickens:
684 Effects of Nutritional State and Dietary Fat. *J Nutr* 117: 428–435, 1987.

- 685 41. **Salichon MR, Guy G, Rousselot D, Blum JC.** Composition des 3 types de foie gras:
686 oie, canard mulard et canard de Barbarie. In: *Annales de zootechnie*. 1994, p. 213–220.
- 687 42. **Saltiel AR, Kahn CR.** Insulin signalling and the regulation of glucose and lipid
688 metabolism. *Nature* 414: 799, 2001.
- 689 43. **Sanyal AJ, Campbell–Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling**
690 **RK, Luketic VA, Shiffman ML, Clore JN.** Nonalcoholic steatohepatitis: Association of
691 insulin resistance and mitochondrial abnormalities. *Gastroenterology* 120: 1183–1192, 2001.
- 692 44. **Schulze RJ, Sathyanarayan A, Mashek DG.** Breaking fat: The regulation and
693 mechanisms of lipophagy. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids* 1862: 1178–
694 1187, 2017.
- 695 45. **Shapira N, Nir I, Budowski P.** Response of lipogenic enzymes to overfeeding in liver
696 and adipose tissue of light and heavy breeds of chicks. *Br J Nutr* 39: 151, 1978.
- 697 46. **Simonen P, Kotronen A, Hallikainen M, Sevastianova K, Makkonen J,**
698 **Hakkarainen A, Lundbom N, Miettinen TA, Gylling H, Yki-Järvinen H.** Cholesterol
699 synthesis is increased and absorption decreased in non-alcoholic fatty liver disease
700 independent of obesity. *J Hepatol* 54: 153–159, 2011.
- 701 47. **Solsona-Vilarrasa E, Fucho R, Torres S, Nuñez S, Nuño-Lámbarri N, Enrich C,**
702 **García-Ruiz C, Fernández-Checa JC.** Cholesterol enrichment in liver mitochondria impairs
703 oxidative phosphorylation and disrupts the assembly of respiratory supercomplexes. *Redox*
704 *Biol* 24: 101214, 2019.
- 705 48. **Tavernier A, Davail S, Ricaud K, Bernadet M-D, Gontier K.** Genes involved in the
706 establishment of hepatic steatosis in Muscovy, Pekin and mule ducks. *Mol Cell Biochem* 424:
707 147–161, 2017.
- 708 49. **Tavernier A, Karine R, Marie-Dominique B, Karine G, Stéphane D.** Pre- and
709 post-prandial expression of genes involved in lipid metabolism at the end of the overfeeding
710 period of mule ducks. *Mol Cell Biochem* 438: 111–121, 2018.
- 711 50. **Uyeda K, Repa JJ.** Carbohydrate response element binding protein, ChREBP, a
712 transcription factor coupling hepatic glucose utilization and lipid synthesis. *Cell Metab* 4:
713 107–110, 2006.
- 714 51. **Vidyasagar A, Wilson NA, Djamali A.** Heat shock protein 27 (HSP27): biomarker of
715 disease and therapeutic target. *Fibrogenesis Tissue Repair* 5: 7, 2012.
- 716 52. **Wu D, Wang X, Zhou R, Cederbaum A.** CYP2E1 enhances ethanol-induced lipid
717 accumulation but impairs autophagy in HepG2 E47 cells. *Biochem Biophys Res Commun*
718 402: 116–122, 2010.
- 719 53. **Yang J, Fernández-Galilea M, Martínez-Fernández L, González-Muniesa P,**
720 **Pérez-Chávez A, Martínez JA, Moreno-Aliaga MJ.** Oxidative Stress and Non-Alcoholic
721 Fatty Liver Disease: Effects of Omega-3 Fatty Acid Supplementation. *Nutrients* 11: 872,
722 2019.
- 723 54. **Yang L, Li P, Fu S, Calay ES, Hotamisligil GS.** Defective Hepatic Autophagy in
724 Obesity Promotes ER Stress and Causes Insulin Resistance. *Cell Metab* 11: 467–478, 2010.
- 725 55. **Yoshida Y, Aoyama Y, Noshiro M, Gotoh O.** Sterol 14-Demethylase P450 (CYP51)
726 Provides a Breakthrough for the Discussion on the Evolution of Cytochrome P450 Gene
727 Superfamily. *Biochem Biophys Res Commun* 273: 799–804, 2000.
- 728 56. **Yu C-L, Yang S-F, Hung T-W, Lin C-L, Hsieh Y-H, Chiou H-L.** Inhibition of
729 eIF2 α dephosphorylation accelerates pterostilbene-induced cell death in human hepatocellular
730 carcinoma cells in an ER stress and autophagy-dependent manner. *Cell Death Dis* 10, 2019.
- 731 57. **Zhu LH, Meng H, Duan XJ, Xu GQ, Zhang J, Gong DQ.** Gene expression profile
732 in the liver tissue of geese after overfeeding. *Poult Sci* 90: 107–117, 2011.

733 58. **Zhuang X, Chung KP, Cui Y, Lin W, Gao C, Kang B-H, Jiang L.** ATG9 regulates
734 autophagosome progression from the endoplasmic reticulum in *Arabidopsis*. *Proc Natl Acad*
735 *Sci* 114: E426–E435, 2017.
736

737
738
739

Table 1a Primers used for determination of mRNA levels

Genes (name and symbol)		Primer sequence 5'-3'
Lipid metabolism		
Acyl CoA dehydrogenase 11 <i>acad11</i>	Forward	TGGTTGTACCTCGAGCTGTG
	Reverse	CATCCACATGAGAGGGGCTTT
ATP Citrate Lyase <i>acly</i>	Forward	ACCCCACTGTTGGACTATGC
	Reverse	GCTTCAAGCGCTTCTGATCT
Acyl CoA Synthetase Long-chain 1 <i>acs11</i>	Forward	GGCTGGCTTCATACAGGAGA
	Reverse	CTCTTTTCTTGGCCCATTTG
Apolipoprotein B <i>apob</i>	Forward	TCTCACCGTGACTTGAGTGC
	Reverse	TCCCAGCAGAAGGTGAAGAT
Apolipoprotein A <i>apoa</i>	Forward	CAAACAGCTCGACCTGAAGC
	Reverse	GGTGTCTTCAGCCACATCT
Cholesteryl Ester Transfer Protein <i>cept1</i>	Forward	CTGCTGTGCAGCTCTTTGAA
	Reverse	CGCAGTATCGAAGCAAATCA
Carnitine Palmitoyl Transferase A <i>cpt1a</i>	Forward	GATTTGGACCAGTGGCTGAT
	Reverse	GAAGGTTGCTTTGCACCAAT
Cytochrome P450 family 5 <i>cyp51a</i>	Forward	CCTAACGCGGTATTTTTGGA
	Reverse	GGCAATGACTTGCTGTCAAA
7-Dehydrocholesterol Reductase <i>dhcr7</i>	Forward	CAAGCATCCTTTCTGCTC
	Reverse	CCTGGAAAGCAACCCAAATA
Diglyceride Acyl Transferase 2 <i>dgat2</i>	Forward	TGGGGCTTGTTACCGTACTC
	Reverse	TGGAGAAGATGGGCTGAATC
Fatty Acid Binding Protein 4 <i>fabp4</i>	Forward	AATGGCTCACTGAAGCAGGT
	Reverse	TGGCTTCTTCATGCCTTTTC
Fatty Acid Synthase <i>fas</i>	Forward	TGAAGAAGGTCTGGGTGGAG
	Reverse	CTCCAATAAGGTGCGGTGAT
Fatty Acid Translocase/Cluster of Differentiation 36 <i>fat/cd36</i>	Forward	AGTTTGCCAAAAGGCTTCAA
	Reverse	CGAGGAACACCACAGAACCT
Glycerol-3-Phosphate Acyltransferase 1 <i>gpai1</i>	Forward	ACAACCTCAGCGGTCCTGTT
	Reverse	GCGCTGAGGTAGGAACGTAG
3-ketoacyl-CoA thiolase, isoform alpha <i>hadh</i>	Forward	ACTTCAGCAAAGCGGTAGGA
	Reverse	ATCCAACCCAACGTAGTCCA
Hydroxymethylglutaryl-CoA Reductase <i>hmgcr</i>	Forward	CATTTTGCTCGTGTCTGGA
	Reverse	ATCCATACTGGCCATTCGAG
Low Density Lipoprotein Receptor <i>ldlr</i>	Forward	TGTGGCCTTCAGAAAGCTCG
	Reverse	ATCTCGTGCTGCATGTAGGG
Lipase Maturation Factor 1 <i>lmf1</i>	Forward	CCTTCCAGACCTACGAGCAG
	Reverse	GCCGATCCTCTTCCGAATCC
Microsomal Triglycerides Transfer Protein <i>mttp</i>	Forward	TGCAGATGGACAGAGTCGAG
	Reverse	GGATGCAGTGCTGAAAACCT
Perilipin 2 <i>plin2</i>	Forward	CAAACCTTCCTTTGGTGAGC
	Reverse	TTGTCCAGACCCATACATGC
Stéaryl CoA désaturase <i>scd1</i>	Forward	AGTGCTGCTCACATGTTTGG
	Reverse	TGAAGTCGATGAAGGCTGTG
Sterol O-acyltransferase 1 <i>soat1</i>	Forward	GCATTCTCAGTTTCGCAAT
	Reverse	TGTGGAGTTCCACCAGTCCT
Very Low Density Lipoprotein Receptor <i>vldlr</i>	Forward	CGTAACTGGCAGCACAAGAA
	Reverse	TGCTGATCCAGTGCTCAAAC

740

741 **Table 1b** Primers used for determination of mRNA levels

Genes (name and symbol)		Primer sequence 5'-3'
Glucids metabolism		
Acetyl CoA Carboxylase	Forward	CATGTTTGAGTGGGCAAAGA
<i>acox</i>	Reverse	TTTTCAGGGCAGGAAAATTG
Enolase Alpha	Forward	CGCTACATGGGGAAAGGTGT
<i>enol</i>	Reverse	AAGGTCAGCAATGTGACGGT
Glyceraldehyde 3 Phosphate Dehydrogenase	Forward	CAGAGGACCAGGTTGTCTCC
<i>gapdh</i>	Reverse	CACCACACGGTTGCTGTATC
Glucose Transporter 1	Forward	CGGGGATCAATGCGGTTTTT
<i>glut1</i>	Reverse	GTGAAGGGTCCCTACGTCCAG
Glucose Transporter 2	Forward	GGAGTTGACCAACCCGTTTA
<i>glut2</i>	Reverse	CCCACCTCGAAGAAGATGAC
Glucose Transporter 3	Forward	TTCTCCAGAAACTCCGTGGC
<i>glut3</i>	Reverse	GGCTCTGTGATACCAGCTCG
Glucose Transporter 8	Forward	ACTGGGAGGCTATCTTGTGG
<i>glut8</i>	Reverse	CAAGAGCCAAGCATAACCAG
Hexokinase 1	Forward	GAGGGAGCAGACGTTGTGAA
<i>hk1</i>	Reverse	GCCGCATCTCCTCCATGTAA
Insuline like Growth Factor 1	Forward	TACCAAGGCATGACCAATGA
<i>igf1</i>	Reverse	AGACCTCTTTGAACGCTGGA
Insulin Receptor	Forward	TGGAGGATACCTGGATCAGC
<i>insr</i>	Reverse	GCAAGTCCTCCTTCAGCATC
Succinate Dehydrogenase Complex, Subunit A	Forward	TGGAACACTGACTTGGTGGA
<i>sdha</i>	Reverse	CCTTGAGTGGCTTGAATA
Transcription factors		
AMP-activated protein kinase	Forward	GCAAGCCTTTTTGATGCTGT
<i>ampk</i>	Reverse	TTCATAAAGGCAGGCTTTGG
Proteine kinase B	Forward	GGGAAGAATGGACGAAAGCC
<i>akt</i>	Reverse	AGTGCCTTTTCCCAGTAGCT
Liver X Receptor Alpha	Forward	CATCTCGACGCTACAATCCA
<i>lxra</i>	Reverse	GTGTGCTGCAGTCTCTCCAC
Mammalian Target of Rapamycin	Forward	ATGGCTTGAAGAGTCGCAGT
<i>mtor</i>	Reverse	AAGGCTTGCAATAGCCAAAA
S6 Protein Kinase 1	Forward	ACTTGGCAAAGGTGGCTATG
<i>s6k1</i>	Reverse	GGGATGTTTTCACTTCCCTCCA
Carbohydrate Response Element Binding Protein	Forward	TCCTCCACACTGCAAAACTG
<i>chrebp</i>	Reverse	ACCATGCCGTTGAAAGACTC
Peroxisome Proliferator Activated Receptor Alpha	Forward	GGATGCTGGTAGCCTATGGA
<i>ppara</i>	Reverse	ACGTGCACAATGCTCTCTTG
Peroxisome Proliferator Activated Receptor Gamma	Forward	CCCAAGTTTGAGTTCGCTGT
<i>pparγ</i>	Reverse	GCTGTGACGACTCTGGATGA
Myocyte Enhancer Factor 2	Forward	CTGGCAACAGCAACACCTAC
<i>mef2</i>	Reverse	GGTGTGGTGGTACGGTCTCT
Hepatocyte Nuclear Factor 4 alpha	Forward	ACCTCAACACCTCCAACAGC
<i>hnf4a</i>	Reverse	ACGCACTGCCTGTAAACCT

743 **Table 1c** Primers used for determination of mRNA levels

Genes (name and symbol)		Primer sequence 5'-3'
Cellular stress		
Asparagine Synthetase	Forward	GGTGATCACGGAAAAGAAGC
<i>asns</i>	Reverse	GTACAGCCCTTTTGGCAGA
Activating Transcription Factor 4	Forward	CAGGTCCTTTTCCTCTGCTG
<i>atf4</i>	Reverse	CCTGGGATTCCCTGGGATACT
Autophagy related gene 4	Forward	AAGGGAAGAGGCAGATGGAT
<i>atg4b</i>	Reverse	CCGCCTGATTTCTTCCATTA
Autophagy related gene 8	Forward	AAATCCCGGTCATCATTGAA
<i>atg8/lc3</i>	Reverse	AGGAAGCCATCCTCATCCTT
Autophagy related gene 9	Forward	TCTACGACGAAGACGTGCTG
<i>atg9</i>	Reverse	CCGCTTTGTACTGGAAGAGC
Caspase 3	Forward	GCAGACAGTGGACCAGATGA
<i>casp3</i>	Reverse	CTTGCAATGTTCCCTCAGCAC
Caspase 8	Forward	GGAAAGCAGTGCCAGAACTC
<i>casp8</i>	Reverse	TAAAATGAAGGGTGCCGAAG
Caspase 9	Forward	CACCCGAAGATGAAACTTGC
<i>casp9</i>	Reverse	CCATGATCCGCTCGACTTAT
Cytochrome P450 2E1	Forward	GCCAGATGCCCTACACAGAT
<i>cyp2e1</i>	Reverse	GGTACCGTTTGATTGAGGA
Heat Shock Protein beta 1	Forward	ACTGGAAGGCGAGAACAAGA
<i>hsbp1</i>	Reverse	TCCGGTAATTCCAAGGGACTG
Sequestosome 1	Forward	AGTCTCCCCGTGATATGGTG
<i>sqstm1/p62</i>	Reverse	ACCATGTTGTGCTCCTTGTG
Reference gene		
<i>luciferase</i>	Forward	CATTCTTCGCCAAAAGCACTCTG
	Reverse	AGCCCATATCCTTGTGCGTATCCC

744

745 **Table 2** Evolution of tissue weights and plasma parameters after 4, 12 and 22 meals (M) (n=32,
746 mean \pm SEM). *AF*: abdominal fat, *SAT*: subcutaneous adipose tissue, *GLU*: glucose, *TG*:
747 triglyceride, *CHO*: cholesterol; ** P <0.01, *** P <0.001, **** P <0.0001
748

	Sampling points			P value
	M4	M12	M22	
Tissues (g)				
Liver	220.9 \pm 3.87 a	344.1 \pm 5.85 b	556.0 \pm 15.12 c	****
Muscle	287.4 \pm 4.43 a	306.9 \pm 4.36 b	314.1 \pm 5.94 b	***
AF	38.09 \pm 2.86 a	77.08 \pm 4.02 b	156.9 \pm 4.25 c	****
SAT	69.59 \pm 2.34 a	116.7 \pm 2.49 b	154.3 \pm 3.05 c	****
Melting rate (%)	-	-	30,884 \pm 17,515	-
Plasma (g/l)				
GLU	3.21 \pm 0.08 a	3.48 \pm 0.11 ab	3.62 \pm 0.07 b	**
TG	3.202 \pm 0.14 a	4.05 \pm 0.20 b	5.27 \pm 0.29 c	****
CHO	1.61 \pm 0.02 a	2.45 \pm 0.04 b	3.06 \pm 0.09 c	****

749

750 **Fig. 1** Correlation level by PCA analysis between tissue weights and plasma parameters in mule
751 ducks (n=96) (A); Correlation level by Pearson correlation analysis between liver weight and
752 plasmatic total cholesterol during overfeeding in mule ducks (n=32 per meal) (B). *AF*:
753 *abdominal fat*, *SAT*: *subcutaneous adipose tissue*, *GLU*: *glucose*, *TG*: *triglyceride*, *CHO*:
754 *cholesterol*

755

756

757

758

759

760

761

762

763

764

765

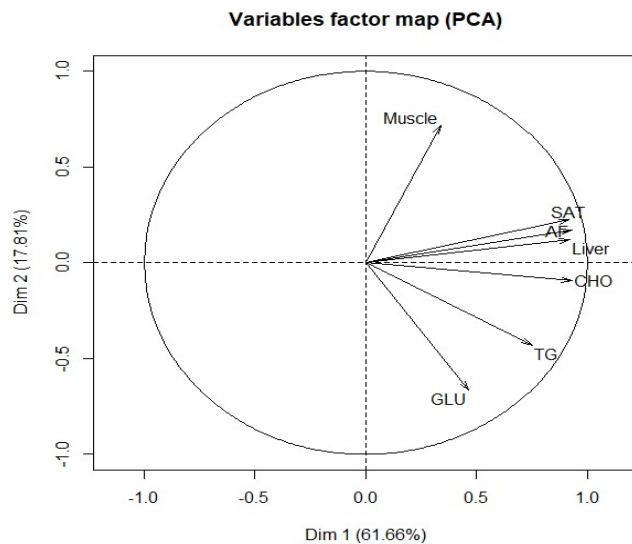
766

767

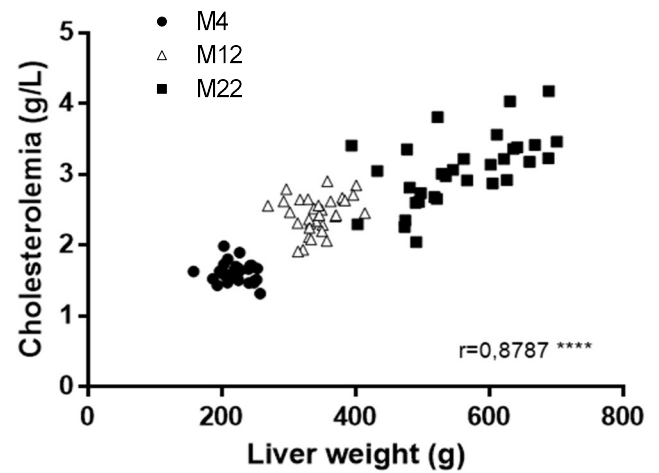
768

769

A



B



770 **Table 3** Relative expression of genes involved in carbohydrate metabolism in liver, muscle and
 771 abdominal fat of mule ducks after 4, 12 and 22 meals (M) (n=32, mean ± SEM); **P < 0.01;
 772 ****P < 0.0001.
 773

Tissue	Target genes	Sampling points			P value
		M4	M12	M22	
Liver					
	<i>hk1</i>	1,80 ± 0,17 a	8,34 ± 1,04 a	40,72 ± 4,92 b	****
	<i>acox</i>	1,46 ± 0,14 a	4,68 ± 0,51 b	7,71 ± 0,65 c	****
	<i>eno1</i>	1,54 ± 0,15 a	1,83 ± 0,15 ab	2,19 ± 0,13 b	**
	<i>sdha</i>	1,37 ± 0,14 a	3,07 ± 0,22 b	4,43 ± 0,35 c	****
	<i>gapdh</i>	1,63 ± 0,18 a	2,77 ± 0,22 a	4,49 ± 0,66 b	****
	<i>glut1</i>	1,08 ± 0,13 a	2,63 ± 0,31 b	6,40 ± 0,62 c	****
	<i>glut2</i>	1,44 ± 0,17 a	2,40 ± 0,22 b	3,39 ± 0,29 c	****
	<i>glut3</i>	1,51 ± 0,19 a	2,79 ± 0,57 a	7,31 ± 0,72 b	****
	<i>glut8</i>	1,27 ± 0,11 a	2,25 ± 0,18 b	3,75 ± 0,26 c	****
	<i>insr</i>	1,37 ± 0,14 a	2,26 ± 0,22 b	3,44 ± 0,24 c	****
	<i>igfl</i>	2,07 ± 0,34 a	6,21 ± 0,87 b	11,39 ± 1,33 c	****
	<i>chrebp</i>	1,50 ± 0,13 a	2,68 ± 0,26 b	3,90 ± 0,36 c	****
Muscle					
	<i>hk1</i>	1,16 ± 0,12	1,40 ± 0,16	1,44 ± 0,11	ns
	<i>acox</i>	1,60 ± 0,21 a	1,75 ± 0,18 a	2,85 ± 0,29 b	***
	<i>eno1</i>	1,57 ± 0,14	1,49 ± 0,15	1,87 ± 0,17	ns
	<i>sdha</i>	1,17 ± 0,08 a	1,20 ± 0,12 ab	1,59 ± 0,15 b	*
	<i>gapdh</i>	1,26 ± 0,08 a	0,89 ± 0,07 b	0,88 ± 0,10 b	**
	<i>glut1</i>	1,08 ± 0,12 a	1,67 ± 0,25 ab	2,12 ± 0,22 b	**
	<i>glut3</i>	1,78 ± 0,23	1,45 ± 0,19	1,97 ± 0,19	ns
	<i>glut8</i>	1,18 ± 0,07 a	1,51 ± 0,13 a	2,24 ± 0,18 b	****
	<i>insr</i>	1,63 ± 0,13	1,75 ± 0,17	2,03 ± 0,16	ns
	<i>igfl</i>	1,71 ± 0,29 ab	1,37 ± 0,21 a	2,29 ± 0,25 b	*
Abdominal fat					
	<i>hk1</i>	0,99 ± 0,09	1,05 ± 0,08	0,93 ± 0,10	ns
	<i>acox</i>	1,10 ± 0,08	1,06 ± 0,08	0,86 ± 0,11	ns
	<i>eno1</i>	1,27 ± 0,10	1,15 ± 0,10	0,97 ± 0,18	ns
	<i>sdha</i>	0,76 ± 0,08 a	0,45 ± 0,03 b	0,42 ± 0,07 b	***
	<i>gapdh</i>	1,25 ± 0,14	1,29 ± 0,11	1,15 ± 0,28	ns
	<i>glut1</i>	1,28 ± 0,11	1,34 ± 0,11	1,12 ± 0,14	ns
	<i>glut3</i>	1,56 ± 0,85	0,53 ± 0,06	0,56 ± 0,10	ns
	<i>glut8</i>	1,15 ± 0,09	1,02 ± 0,06	1,03 ± 0,11	ns
	<i>insr</i>	1,33 ± 0,16 a	0,84 ± 0,08 b	0,82 ± 0,14 b	**
	<i>igfl</i>	1,86 ± 0,39	1,20 ± 0,18	1,33 ± 0,33	ns
	<i>chrebp</i>	1,78 ± 0,23 a	0,78 ± 0,13 b	1,62 ± 0,38 ab	*

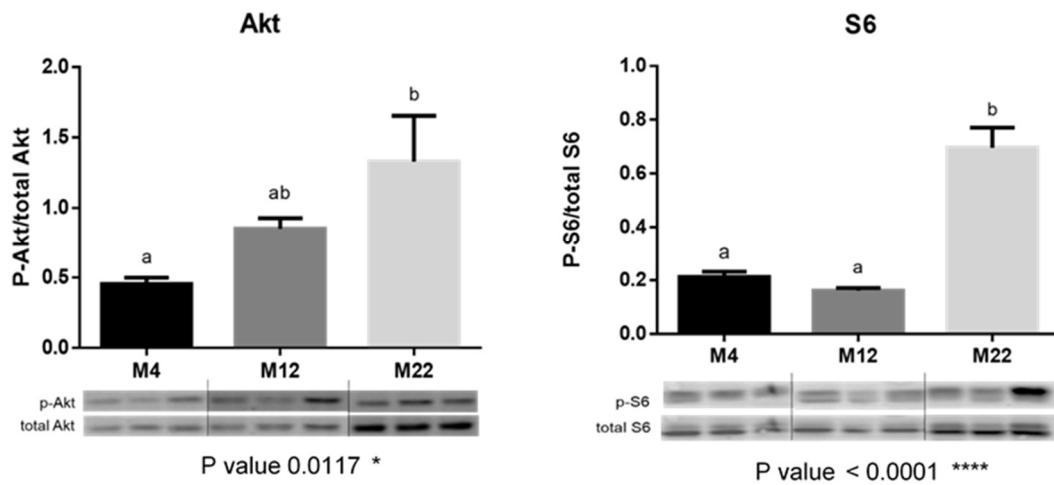
774

775 **Table 4** Relative expression of genes involved in lipid metabolism in liver, muscle and
 776 abdominal fat of mule ducks after 4, 12 and 22 meals (M) (n=32, mean ± SEM); **P < 0.01;
 777 ****P < 0.0001.

Tissue	Target genes	Sampling points			P value
		M4	M12	M22	
Liver					
Lipogenesis	<i>fas</i>	1,25 ± 0,11 a	1,85 ± 0,13 b	1,73 ± 0,12 b	**
	<i>acsl1</i>	1,31 ± 0,13 a	3,33 ± 0,30 b	6,28 ± 0,68 c	****
	<i>dgat2</i>	2,34 ± 0,41 a	6,60 ± 0,77 b	9,10 ± 1,54 b	****
	<i>scd1</i>	2,45 ± 0,41 a	7,93 ± 1,05 b	13,45 ± 2,12 c	****
	<i>acly</i>	1,17 ± 0,12	1,20 ± 0,11	1,23 ± 0,10	ns
β oxidation	<i>cpt1a</i>	2,10 ± 0,35 a	9,52 ± 0,99 a	39,35 ± 4,88 b	****
	<i>acad</i>	2,14 ± 0,30 a	12,60 ± 1,77 b	17,49 ± 3,10 b	****
	<i>hadh</i>	1,73 ± 0,21 a	4,37 ± 0,44 b	5,55 ± 0,53 b	****
Lipoprotein formation	<i>apob</i>	2,02 ± 0,22 a	4,39 ± 0,38 b	10,32 ± 0,96 c	****
	<i>apoa</i>	1,36 ± 0,18 a	3,11 ± 0,37 b	3,63 ± 0,25 b	****
	<i>mtp</i>	1,46 ± 0,16 a	2,32 ± 0,15 b	2,38 ± 0,27 b	**
	<i>cept1</i>	1,90 ± 0,25 a	4,32 ± 0,44 b	6,99 ± 0,67 c	****
	<i>plin2</i>	1,54 ± 0,18 a	3,58 ± 0,26 b	6,96 ± 0,56 c	****
	<i>gpat1</i>	1,38 ± 0,14 a	3,65 ± 0,32 b	3,60 ± 0,22 b	****
	<i>lmf1</i>	1,47 ± 0,19 a	2,89 ± 0,37 b	4,13 ± 0,43 c	****
Fatty acid maturation and transport	<i>fabp4</i>	2,63 ± 0,53 a	100,44 ± 18,55 a	2874,26 ± 395,59 b	****
	<i>fat/cd36</i>	1,98 ± 0,23 a	6,97 ± 0,76 b	10,51 ± 0,92 c	****
Lipoprotein receptors	<i>vldlr</i>	1,65 ± 0,19 a	5,00 ± 1,01 b	9,15 ± 0,74 c	****
	<i>ldlr</i>	1,43 ± 0,22 a	3,07 ± 0,27 b	4,67 ± 0,41 c	****
Cholesterol synthesis and esterification	<i>hmgcr</i>	4,00 ± 0,35 a	5,44 ± 0,54 a	7,76 ± 0,92 b	***
	<i>dher7</i>	3,01 ± 0,35	4,57 ± 0,58	3,46 ± 0,54	ns
	<i>cyp51a</i>	1,89 ± 0,27 a	2,68 ± 0,39 b	1,59 ± 0,16 a	*
	<i>soat1</i>	1,53 ± 0,15 a	3,63 ± 0,31 b	8,50 ± 0,78 c	****
Transcription factors	<i>ppara</i>	1,54 ± 0,19 a	5,85 ± 0,52 b	8,84 ± 0,79 c	****
	<i>ppary</i>	1,47 ± 0,15 a	5,84 ± 0,56 b	9,94 ± 1,12 c	****
	<i>lxra</i>	1,69 ± 0,20 a	5,58 ± 0,46 b	8,52 ± 0,72 c	****
Muscle					
Lipogenesis	<i>fas</i>	1,01 ± 0,20	1,06 ± 0,20	1,29 ± 0,27	ns
	<i>acsl1</i>	1,18 ± 0,06 a	2,99 ± 0,35 b	3,23 ± 0,32 b	****
	<i>dgat2</i>	1,17 ± 0,15	1,70 ± 0,27	1,68 ± 0,20	ns
	<i>scd1</i>	1,57 ± 0,35	1,67 ± 0,30	2,30 ± 0,61	ns
	<i>acly</i>	1,16 ± 0,20 ab	1,03 ± 0,10 a	1,72 ± 0,25 b	*
β Oxidation	<i>cpt1a</i>	1,64 ± 0,24 a	2,37 ± 0,29 a	3,82 ± 0,30 b	****
	<i>acad</i>	1,65 ± 0,21 a	2,21 ± 0,36 ab	3,38 ± 0,57 b	*
	<i>hadh</i>	1,46 ± 0,14 a	2,38 ± 0,31 ab	3,28 ± 0,57 b	***
Lipoprotein formation	<i>apob</i>	2,05 ± 0,64	1,82 ± 0,42	2,63 ± 0,67	ns
	<i>apoa</i>	1,28 ± 0,19	0,93 ± 0,15	1,45 ± 0,23	ns
	<i>mtp</i>	0,72 ± 0,19	0,61 ± 0,15	0,81 ± 0,21	ns
	<i>cept1</i>	1,12 ± 0,17 a	1,20 ± 0,12 a	1,77 ± 0,25 b	*
	<i>plin2</i>	1,12 ± 0,08 ab	0,94 ± 0,06 a	1,41 ± 0,13 b	**
	<i>gpat1</i>	1,49 ± 0,33 a	2,75 ± 0,45 ab	3,14 ± 0,50 b	*
	<i>lmf1</i>	1,68 ± 0,18 a	2,31 ± 0,30 ab	3,60 ± 0,34 b	****
Fatty acid maturation and transport	<i>fabp4</i>	1,03 ± 0,35	1,04 ± 0,30	1,52 ± 0,20	ns
	<i>fat/cd36</i>	1,64 ± 0,18 a	1,64 ± 0,19 a	2,68 ± 0,26 b	***
Lipoprotein receptors	<i>vldlr</i>	1,43 ± 0,08 a	1,79 ± 0,12 ab	1,99 ± 0,14 b	**
	<i>ldlr</i>	1,17 ± 0,22	0,93 ± 0,12	1,10 ± 0,09	ns
Cholesterol esterification	<i>soat1</i>	1,59 ± 0,21	1,27 ± 0,17	1,91 ± 0,24	ns

<i>Transcription factors</i>	<i>ppara</i>	1,3 ± 0,13 a	2,76 ± 0,39 b	3,25 ± 0,33 b	****
	<i>ppary</i>	0,97 ± 0,11	0,65 ± 0,12	0,79 ± 0,07	ns
	<i>lxra</i>	1,31 ± 0,12 a	1,75 ± 0,19 ab	2,30 ± 0,18 b	***
Abdominal fat					
	<i>acsll</i>	1,04 ± 0,10 a	0,75 ± 0,06 ab	0,56 ± 0,10 b	**
	<i>apoa</i>	0,97 ± 0,10 a	0,44 ± 0,05 b	0,99 ± 0,18 a	**
	<i>lmfl</i>	1,19 ± 0,15 ab	1,45 ± 0,18 a	0,82 ± 0,11 b	*

779 **Fig. 2** Western blot analysis of hepatic protein kinase (Akt), ribosomal protein S6 (S6)
780 phosphorylation in mule ducks after 4, 12 and 22 meals (M). A representative blot is shown.
781 Graphs represent the ratio between the phosphorylated protein and the total amount of the target
782 protein (n=3, mean \pm SEM).



783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805

806 **Table 5** Relative expression of genes involved in mTOR pathway in liver, muscle and
 807 abdominal fat of mule ducks after 4, 12 and 22 meals (M) (n=32, mean \pm SEM); **P < 0.01;
 808 ****P < 0.0001.
 809

Tissue	Target genes	Sampling points			P value
		M4	M12	M22	
Liver					
	<i>akt</i>	1,24 \pm 0,11 a	2,92 \pm 0,19 b	4,14 \pm 0,26 c	****
	<i>mtor</i>	1,43 \pm 0,12 a	3,27 \pm 0,30 b	5,16 \pm 0,56 c	****
	<i>s6kl</i>	1,40 \pm 0,15 a	3,71 \pm 0,27 b	6,34 \pm 0,59 c	****
Muscle					
	<i>akt</i>	1,13 \pm 0,11	1,20 \pm 0,11	1,39 \pm 0,09	ns
	<i>mtor</i>	1,07 \pm 0,09	1,12 \pm 0,10	1,12 \pm 0,08	ns
	<i>s6kl</i>	1,22 \pm 0,12 a	1,50 \pm 0,16 ab	1,83 \pm 0,16 b	*
Abdominal fat					
	<i>akt</i>	1,08 \pm 0,08	1,06 \pm 0,07	0,92 \pm 0,11	ns
	<i>mtor</i>	0,91 \pm 0,08	0,71 \pm 0,06	0,70 \pm 0,09	ns
	<i>s6kl</i>	1,14 \pm 0,09	0,86 \pm 0,06	0,86 \pm 0,11	ns

810

811 **Table 6** Relative expression of genes involved in global cellular stress in liver, muscle and
 812 abdominal fat of mule ducks after 4, 12 and 22 meals (M) (n=32, mean ± SEM); **P < 0.01;
 813 ****P < 0.0001.

Tissue	Target genes	Sampling points			P value
		M4	M12	M22	
Liver					
Amino acid deficiency	<i>asns</i>	1,83 ± 0,25 a	8,13 ± 0,97 b	13,07 ± 1,52 c	****
	<i>atf4</i>	1,39 ± 0,14 a	4,14 ± 0,34 b	7,87 ± 0,69 c	****
Autophagy	<i>atg4b</i>	1,77 ± 0,20 a	4,43 ± 0,37 b	6,51 ± 0,54 c	****
	<i>atg8 / lc3</i>	3,40 ± 0,59 a	9,97 ± 0,97 b	23,08 ± 2,10 c	****
	<i>atg9</i>	1,28 ± 0,17 a	4,04 ± 0,46 b	6,62 ± 0,86 c	****
	<i>sqstm1/ p62</i>	1,58 ± 0,21 a	3,43 ± 0,33 b	6,30 ± 0,57 c	****
	<i>lamp2a</i>	1,46 ± 0,14 a	4,64 ± 0,34 b	6,96 ± 0,47 c	****
Cellular stress	<i>cyp2e1</i>	2,10 ± 0,40 a	9,50 ± 1,53 b	11,97 ± 2,24 b	****
	<i>hsbp1</i>	1,56 ± 0,20 a	5,35 ± 0,47 b	9,72 ± 0,91 c	****
Apoptosis	<i>casp3</i>	1,99 ± 0,23 a	5,49 ± 0,53 b	12,01 ± 1,09 c	****
	<i>casp8</i>	1,38 ± 0,13 a	2,93 ± 0,20 b	5,14 ± 0,32 c	****
	<i>casp9</i>	1,37 ± 0,16 a	4,16 ± 0,46 b	7,06 ± 0,55 c	****
Muscle					
Amino acid deficiency	<i>asns</i>	1,19 ± 0,14 a	1,64 ± 0,27 a	2,51 ± 0,30 b	**
	<i>atf4</i>	4,62 ± 1,99	7,76 ± 2,69	8,26 ± 2,56	ns
Autophagy	<i>atg4b</i>	1,57 ± 0,12 a	2,07 ± 0,21 ab	2,27 ± 0,18 b	*
	<i>atg8 / lc3</i>	3,75 ± 1,29	2,56 ± 0,30	3,60 ± 0,30	ns
	<i>atg9</i>	2,61 ± 0,73	2,44 ± 0,28	2,49 ± 0,32	ns
	<i>sqstm1/ p62</i>	1,36 ± 0,15	1,49 ± 0,12	1,56 ± 0,12	ns
	<i>lamp2a</i>	1,19 ± 0,09	1,09 ± 0,09	1,19 ± 0,08	ns
Cellular stress	<i>cyp2e1</i>	1,16 ± 0,12	1,40 ± 0,16	1,44 ± 0,11	ns
	<i>hsbp1</i>	1,20 ± 0,17 a	1,67 ± 0,26 ab	2,18 ± 0,22 b	**
Apoptosis	<i>casp3</i>	1,17 ± 0,12 a	2,18 ± 0,33 b	2,61 ± 0,29 b	***
	<i>casp8</i>	1,17 ± 0,08 a	1,57 ± 0,18 ab	2,07 ± 0,19 b	***
	<i>casp9</i>	1,34 ± 0,17 a	1,96 ± 0,27 a	2,90 ± 0,32 b	***
Abdominal fat					
Amino acid deficiency	<i>asns</i>	0,96 ± 0,21	0,69 ± 0,07	0,69 ± 0,15	ns
	<i>atf4</i>	1,25 ± 0,53	0,64 ± 0,06	0,62 ± 0,10	ns
Autophagy	<i>atg4b</i>	1,30 ± 0,17	1,15 ± 0,10	0,94 ± 0,17	ns
	<i>atg8 / lc3</i>	1,43 ± 0,16	1,44 ± 0,14	1,51 ± 0,25	ns
	<i>atg9</i>	2,03 ± 0,38	1,67 ± 0,29	1,06 ± 0,16	ns
	<i>sqstm1/ p62</i>	1,13 ± 0,33	0,77 ± 0,08	0,80 ± 0,11	ns
	<i>lamp2a</i>	1,17 ± 0,11	1,08 ± 0,08	1,06 ± 0,15	ns
Cellular stress	<i>cyp2e1</i>	1,65 ± 0,47	5,02 ± 1,29	8,43 ± 3,82	ns
	<i>hsbp1</i>	1,18 ± 0,12	1,30 ± 0,09	1,32 ± 0,25	ns
Apoptosis	<i>casp3</i>	1,12 ± 0,09	0,99 ± 0,07	0,96 ± 0,17	ns
	<i>casp8</i>	1,15 ± 0,08 a	0,95 ± 0,06 ab	0,81 ± 0,09 b	*
	<i>casp9</i>	3,07 ± 2,59	0,47 ± 0,05	0,35 ± 0,06	ns

814

815 **Fig 3.** Correlation level by Pearson correlation analysis between FABP4 mRNAs expression
816 and the melting rate achieved at the end of overfeeding (M22) in mule ducks (n=32).

817

818

