PAPER I

1 **Development and characterisation of some intestinal enzymes in Atlantic**

2 **halibut (<u>Hippoglossus hippoglossus</u> L.) and Atlantic cod (<u>Gadus morhua</u> L.)**

3 **larvae.**

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17

18   **Abstract**

19

20   To determine the status of development of the intestine, the activities of intestinal leucine-

21   alanine peptidase (Leu-ala), brush border membrane (bbm)-bound alkaline phosphatase (AP)

22   and bbm-bound leucine aminopeptidase N (LAP) were analysed from first feeding to passed

23   weaning in Atlantic halibut (Hippoglossus hippoglossus; 1-78 days post first feeding; dpff)

24   and Atlantic cod (Gadus morhua; 0-72 dpff). Before conducting the ontogenetic studies, the

25   enzyme assays used were optimised in relation to temperature and pH (only AP and LAP),

26   and the Michaelis constant (K_M; All enzymes) determined, in order to ensure valid data and to

27   maximise the activity potentials. Leu-ala was used as an indicator of pinocytotic activity

28   which was expected to decrease during the experimental periods. AP and LAP were used as

29   indicators of development of the bbm and achievement of an adult mode of digestion. In

30   halibut, Leu-ala increased 11 folds ($\underline{P}$=0.0002) in specific activity (mU (mg protein)$^{-1}$) from

31   first feeding to 34 dpff and thereafter a small decrease (0.65-fold; $\underline{P}$= 0.006) in activity was

32   observed. In cod, the specific activity of Leu-ala varied randomly during the experiment.

33   Distinct increases in specific activities of AP and LAP were observed from 29 to 52 dpff in

34   halibut (fivefold; $\underline{P}$<0.002) and from 29 to 37 dpff in cod (two-threefold; $\underline{P}$<0.02). The bbm-

35   bound activities of AP and LAP in percent of total intestinal activity of the respective enzyme

36   increased from approximately 2 to 15% between 34 and 43 dpff in halibut ($\underline{P}$<0.0003),

37   confirming the results of specific activities. In cod, a similar result with AP was found

38   between 29 and 51 dpff ($\underline{P}$=0.0003). The increase in percentage of bbm-bound activity of

39   LAP was later, increasing exponentially from constituting 5 to 31 % of total individual

40   activity between 37 and 72 dpff ($\underline{P}$=0.0003). The experiments indicated that the digestion in

41   halibut had advanced towards the adult mode at 40 to 50 dpff, while time of reaching the

42   same maturational level in cod was less certain due to the different activity profiles of AP and

43   LAP. As AP is well recognised as a bbm differentiation marker, it can be assumed that the

44   intestine in cod matured when AP activity had reached an elevated level at approximately 40

45   to 50 dpff, wheras LAP possibly was induced later. Ceasing of the larval mode of digestion,

46   expected to be indicated by reduced activity of Leu-ala, was not detected.

47

48   *Keywords*: Alkaline phosphatase; Aminopeptidase; Atlantic cod; Atlantic halibut; Intestine;

49   Larvae

50

51   **1. Introduction**

52

53   A characteristic pattern of development of the digestive tract has been described in marine

54   fish larvae which do not have a functional stomach at first feeding (Zambonino Infante and

55   Cahu, 2001). Before the stomach is developed, luminal protein digestion depends on action of

56   secreted pancreatic enzymes. In addition, endogenous enzymes or other digestive stimulators

57   from the live prey might promote larval digestion (Kolkovski, 2000). Furthermore,

58   intracellular digestion of pinocytotically absorbed protein might be of nutritional importance

59   in this pre-gastric period (Watanabe, 1982). By pinocytosis, proteins and larger peptides are

60   absorbed by the enterocytes in the hindgut, and then assumed degraded by lysosomal

61   cathepsins (Davies and Messer, 1984; Sire and Vernier, 1992). Due to their similar

62   ontogenetic development as cathepsins in the distal intestine in suckling rat, some cytosolic

63   dipeptidases have been proposed to be involved in the terminal process of intracellular

64   digestion when lysosomal peptides are released into the cytosol (Vaeth and Henning, 1982;

65   Henning, 1987). In marine fish larvae, decline in the activity of leucine-alanine peptidase

66   (Leu-ala) is found during the pre-gastric period (Ribeiro et al., 1999; Zambonino Infante and

67   Cahu, 2001), suggesting similar processes in marine fish larvae as has been described to take

68   place in the suckling rats.

69

70   At the end of the larval period, pinocytosis diminishes, digestive capacity in the gut lumen

71   and nutrient absorption improves and gastric digestion becomes established (Pedersen and

72   Falk-Petersen, 1992; Luizi et al., 1999; Ribeiro et al., 1999; Zambonino Infante and Cahu,

73   2001). The brush border membrane (bbm) enzymes leucine aminopeptidase N (LAP) and

74   alkaline phosphatase (AP) abruptly increase in activity during the course of the larval stage

75   and indicate the time when the intestine matures and attains a more adult character with

76   improved luminal digestive capacity (Ribeiro et al., 1999; Zambonino Infante and Cahu,

77   2001).

78

79   The morphological and histological development of the digestive tract in larval halibut

80   (Hippoglossus hippoglossus) and cod (Gadus morhua) have been described (Kjørsvik et al.,

81   1991; Pedersen and Falk-Petersen, 1992; Kjørsvik and Reiersen, 1992; Luizi et al., 1999),

82   whereas their digestive capacities are less clear, especially in halibut, although some studies

83   have dealt with this issue (Hjelmeland et al., 1984; Gawlicka et al., 2000; Rojas-García et al.,

84   2002; Perez-Casanova et al., 2006).

85

86   The primary aim of the present study was to describe the development of the three intestinal

87   enzymes AP, LAP and Leu-ala from first feeding until passed weaning in larval halibut and

88   cod. These enzymes were selected because they in other species have indicated a significant

89   step in intestinal maturation that might be important for readiness of formulated diet

90   introduction (Ribeiro et al., 1999; Zambonino Infante and Cahu, 2001). The two former

91 enzymes were expected to have a sudden increase at a specific time during the experimental

92 period, while the latter one was expected to decrease progressively.

93

94 Secondly, the current study also aimed to determine the optimum temperatures and pH-values

95 and the Michaelis constants (K_M) to the enzymes studied in order to optimise the assay

96 conditions prior to the enzymatic studies. Optimisation of the assays will ensure a linear

97 product release during the assay-course, which is demanded for producing valid quantitative

98 data, and maximise the enzyme activity determined and thus give clearer differences between

99 experimental groups.

100

101

102 **2. Materials and Methods**

103

104 *2.1. Biological material for the enzyme optimisation study*

105

106 Juvenile halibut and cod were collected at the Institute of Marine Research, Storebø, Norway,

107 where they had been fed a heat-coagulated, protein-bound microdiet (Hamre et al. 2001). The

108 wet weight of the halibut at the sampling day was 10.6±0.4 g and the age was approximately 6

109 month after first feeding. The same data for cod were 0.31±0.06 g and 82 days post hatch

110 (dph), respectively. At sampling, the fish were immediately frozen on dry ice and kept at -

111 80ºC until dissection. The intestines (from the pyloric sphincter to the anus) were dissected

112 from semi-thawed individuals for use in preparation of enzyme extract.

113

114 *2.2. Sample preparation for enzyme assays*

115   The sample preparation and enzyme assay methods were in accordance to Cahu et al., (1999).

116   The samples were homogenised (Polytron PT2100, Kinematica AG, Lucerne, Switzerland) in

117   30 v/w fractions of Tris(2 mM)-Mannitol(50 mM), pH 7 for 30 sec at 22 000 rpm. To prepare

118   the bbm extracts as described by Crane et al., (1979), the homogenates were centrifuged at 9

119   000g for 10 min after addition of 0.1 M $CaCl_2$. The supernatants were transferred to new vials

120   and further centrifuged at 34 000g for 20 min. The precipitated bbm were dissolved in Tris(5

121   mM)-HEPES(5 mM)-KCl(10 mM)-DTT(1 mM), pH 7.5. All steps in the sample preparations

122   described above were conducted at low temperature by working on ice. The enzyme extracts

123   were stored frozen (-80°C) until analysis of enzyme activity or protein content.

124

125   *2.3. Enzyme and protein assays*

126

127   AP (EC 3.1.3.1) and LAP (EC 3.4.11.1) activities were determined in the bbm extracts and in

128   the homogenates, while the activity of Leu-ala was determined only in the homogenates.

129

130   The method for analysing activity of AP is based on the method described by Bessey et al.,

131   (1946). The substrate used was *p*-nitro-phenyl-phosphate (7 mM; Sigma-Aldrich Corp.,

132   St.Louis, Mo, USA) dissolved in 30 mM Na_2CO_3/$NaHCO_3$ added 1 mM $MgCl_2$. The buffer

133   pH was 9.8 when determining temperature optimum and 10.3 and 10.1 for halibut and cod,

134   respectively, when determining K_M. Enzyme activity was monitored spectrophotometrically

135   (Shimatzu Hyperuv-1601PC, ver 1.5, Shimatzu Deutchland GmbH, Duisburg, Germany) for 2

136   min at 407 nm (30±1ºC and 37±1ºC for halibut and cod, respectively) from the time of

137   addition of enzyme extract. Linear product release during the assay-course was checked by

138   automatic registration of absorbance every 20 seconds. When departure from linearity

139   sometimes was observed, the deviating 20 second interval was not included in the calculation

140   of activity, or the sample was reanalysed. The molar extinction coefficient of 18 300 $M^{-1}cm^{-1}$

141   was used in calculation of the enzyme activity.

142

143   The substrate for analysing the amidolytic activity of LAP was 2 mM leucine-*p*-nitroanilide

144   (Sigma-Aldrich Corp., St.Louis, Mo, USA; Maroux et al., 1973). The activity was measured

145   in 80 mM NaH_2PO_4/Na_2HPO_4, pH 7.0 when determining temperature optimum and 8.1 and

146   7.2 for halibut and cod, respectively, when determining K_M. Product release was monitored

147   for 2 minutes at 410 nm and 37±1ºC from the time of addition of enzyme extract. Linear

148   product release was ensured as for AP. Enzyme activity was calculated using the molar

149   extinction coefficient of 8 200 $M^{-1}cm^{-1}$.

150

151   Leu-ala activity was determined in a coupled assay with L-amino acid oxidase (EC 1.4.3.2;

152   Sigma-Aldrich Corp., St.Louis, Mo, USA) and horseradish peroxidase (EC 1.11.1.7; Sigma-

153   Aldrich Corp., St.Louis, Mo, USA) as auxiliary enzymes and *o*-dianisidine as the

154   chromophore, all added in excess (Nicholson and Kim, 1975). Substrate and buffer used was

155   L-leucyl-L-alanine (3.125 mM; Sigma-Aldrich Corp., St.Louis, Mo, USA) and 50 mM Tris-

156   HCl, (pH 8), respectively. Activity was initiated by addition of substrate in the cuvette and

157   terminated by addition of 1.74 ml 50% H_2SO_4 after 20 minutes incubation at 25±1ºC.

158   Concentration of product (oxidised *o*-dianisidine) was measured at 530 nm. Leucine (Sigma-

159   Aldrich Corp., St.Louis, Mo, USA) was used as standard.

160

161   Protein content of homogenates and bbm extracts was determined by Bio-Rad Protein

162   microassay (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as standard.

163

164   *2.4. Assay optimisation*

165

166 For determination of temperature optimum, enzyme activity measurements were performed at

167 intervals of 1-3 ºC in a range of approximately ±10ºC of the optimal values found. To

168 determine the pH optimum, enzyme activity measurements were performed at intervals of

169 about 0.2 pH units in a range of about ±0.5-1 pH units from the measured optimum. The

170 Michaelis constants (K_M) were determined by varying the substrate concentrations from 0.2 to

171 14.5 mM, from 0.02-0.1 to 2.4 mM and from 0.06 to 1.56-4.69 mM in the AP, LAP and Leu-

172 ala assays, respectively. These concentrations corresponded at a minimum to 0.8 K_M -10 K_M

173 (Tab 1). K_M was obtained by direct linear plots as described by Henderson (1992). In short,

174 for each pair of ([S], V) measured ([S]=substrate concentration, V=specific enzyme activity),

175 -[S] is plotted on the horizontal K_M axis, V is plotted on the vertical V_{max} axis and a line is

176 drawn through these two points. Hence, n measurements give rise to n lines that will intersect.

177 K_M is defined by the coordinate of the median intersection (or the mean of two medians) of

178 these lines on the K_M axis. In all optimisation studies, measurements were made either

179 singular or in duplicates with a total of 13-32 measurements. The exception was Leu–ala in

180 halibut where 8 measurements were used.

181

182 *2.5. Rearing conditions for halibut and cod in the ontogenetic study*

183

184 For determining the development of enzyme activities in Atlantic halibut throughout the

185 larval period, larvae were obtained from a commercial hatchery (Nordic Seafarms, Askøy,

186 Norway). The larvae were first fed at 253 daydegrees (43 days after hatch) in 11 000 L

187 rearing tanks at 11-12º C (gradual increase in temperature from 6ºC within the first week).

188 They were fed <u>Artemia</u> (INVE Aquaculture nv, Dendermonde, Belgium) enriched with

189 Multigain (Danafeed, Horsens, Denmark) from 0 to 74 days post first feeding (dpff) and

190   thereafter a commercial formulated diet (Danafeed, Horsens, Denmark) to the end of the study

191   (78 dpff). From first feeding to 45 dpff, turbidity in the rearing water was increased by

192   addition of clay (Waldm. Ellefsen AS, Oslo, Norway), a procedure with similar effect as

193   green water obtained by addition of algae.

194

195   For determination of the ontogeny of digestive enzymes in cod, cod larvae were reared at the

196   experimental facilities at the Institute of Marine Research, Storebø, Norway as described by

197   van der Meeren and Ivannikov (2006). Eggs, collected from one group of broodstock, were

198   purchased from a commercial hatchery. Larvae were first-fed 3 days after hatching and reared

199   in 500 L tanks at 11-12 ℃ (gradual increase from 8℃ within the first days). They were fed

200   rotifers (Brachionus plicatilis) grown on baker's yeast (Idun Industri AS, Oslo, Norway) and

201   Rotimac (Biomarine Aquafauna Inc.,Hawthorne, CA, USA) from 0 to 40 dpff, Artemia

202   (Artemia Systems Inc., Gent, Belgium) enriched with DC DHA Selco (INVE Aquaculture

203   NV, Dendermonde, Belgium) from 35 to 50 dpff, and a commercial formulated diet

204   (Aglonorse, Ewos AS, Bergen, Norway) from 50 dpff to the end of the experiment (72 dpff).

205   Green water was used from first feeding to 35 dpff by adding Instant Algae®

206   (Nannochloropsis; Reed Mariculture, Campbell, CA, USA) to the rearing tanks to keep a

207   turbidity of approximately 1-2 NTU.

208

209   *2.6. Sampling, sample preparation and enzyme assays in the ontogenetic studies*

210

211   Sample collections were conducted before morning feed distribution in the study with halibut,

212   whereas cod was by a mistake collected after feeding. Halibut larvae were sampled at 1, 8, 14,

213   22, 29, 34, 43, 52, 58, 65, 71 and 78 dpff and cod larvae were sampled at 0, 7, 14, 22, 29, 37,

214   43, 51, 59, 66 and 72 dpff. At the last sampling day of halibut, two samples were collected.

215   One sample included the largest individuals that had juvenile pigmentation pattern and settled

216   behaviour, and one sample included the smallest individuals which behaved more pelagic and

217   had less mature pigmentation pattern. At sampling, the larvae were anaesthetised by metacain

218   (approximately 0.5-1 mg L^{-1}) and then immediately frozen on dry ice and kept at -80ºC until

219   preparation of enzyme extract or measurement of larval size.

220

221   For registration of standard length (SL; halibut and cod) and wet weight (halibut), 10-50

222   larvae were used. Estimates of specific growth rate (SGR; % day^{-1}) of halibut were based on

223   wet weights and calculated as: SGR = (e^g -1)*100%, where g = (ln(final weight)-ln(initial

224   weight))/experimental days (Houde and Schekter, 1981).

225

226   For the enzyme activity determinations, 3 pooled samples of larvae were collected at each

227   sampling and the number of larvae in each sample varied from 10 to 300 depending on larval

228   size. In the experiment with halibut, the segment of the larvae containing the abdominal

229   cavity (*i.e.* from end of the operculum to the anus) was used in the enzyme extract

230   preparations. This segment is in the current study notes as the intestinal segment of the larvae,

231   and is a slight modification of the practice in other studies dealing with ontogenetic changes

232   in intestinal enzymes (Cahu and Zambonino Infante, 1994; Cahu et al., 1999; Ribeiro et al.,

233   1999; Ma et al., 2005). In the samples of halibut from 29 dpff and onwards, the dorsal part of

234   the segments were also removed. In the experiment with cod, whole larvae were used in the

235   preparations of the five first samplings (0-29 dpff), while the intestinal segments of the larvae

236   were used in the remaining cod sample preparations. This change from using whole larvae to

237   the intestinal segment of the larvae coincided with marked changes in enzyme activities (See

238   results). For AP and LAP, reanalyses of homogenates of both whole larvae and intestinal

239   segments at the three sample dates after the shift in method (37-41 dpff) confirmed that the

240   activities of these enzymes mainly were present in the intestinal segment of the larvae (results

241   not shown). Thus, AP and LAP activities are presented in the figures with unbroken lines,

242   whereas the Leu-ala activity profiles are given by the line broken between 29 and 37 dpff.

243

244   Enzyme extracts were prepared as described in section 2.2 and enzyme assays were run as

245   described in section 2.3 with the following temperature and pH specifications during assay

246   run: AP activity was analysed at pH 10.3 and 10.1, and temperatures 30 ± 1 and $37\pm1°C$ in the

247   assays of halibut and cod, respectively. LAP activity was analysed at pH 8.1 and 7.2, in the

248   assays of halibut and cod, respectively, and at $37\pm1°C$ for both species. Leu-ala activity was

249   analysed at pH 8 and $25\pm1°C$.

250

251   *2.6. Statistics*

252

253   Statistical analyses were conducted by Statistica (version 6.1; StatSoft Inc., Tulsa, OK, USA).

254   Repeated measures ANOVA followed by Tukey HSD was used to evaluate statistical

255   differences between the enzyme activity at different developmental stages. The data on

256   individual activity and activity in bbm in percent of total activity were $\log_{10}$ transformed prior

257   to analysis. Regression equations for exponential or linear increases in growth or enzyme

258   activity were calculated when such increases were found during more than two samplings.

259   The corresponding coefficient of determination (R^2) and significance level of the slope are

260   given. The analyses are based on means $\pm$ SD of three replicates, and the data were

261   considered significantly different when $p < 0.05$.

262

263 **3. Results**

264

265 *3.1. Enzyme characteristics*

266

267 The optimum temperature and pH for the enzymatic activities of AP and LAP are listed in

268 Table 1. Temperature optimum for AP activity was 30 ± 1ºC and 34 ± 1ºC in halibut and cod,

269 respectively, when assayed at pH 9.8 ± 0.1. Rising the temperature to 37 ± 1ºC, reduced the

270 activity to 53 and 95% of the maximum in halibut and cod, respectively. LAP was more

271 resistant to heat denaturation in both species and showed temperature optimum of 42 ± 1ºC and

272 47 ± 1ºC in halibut and cod, respectively, when assayed at pH 7 ± 0.1. The activity at 37 ± 1ºC

273 was 88 and 69% of the maximum in halibut and cod, respectively. The pH values giving the

274 highest activity of AP were 10.3 ± 0.1 and 10.1 ± 0.1 in halibut (temperature 30 ± 1ºC) and cod

275 (temperature 37 ± 1ºC), respectively. At pH 9.8 ± 0.1 the activity was reduced to 74 and 90% of

276 the maximum in halibut and cod, respectively. LAP differed more in optimal pH between the

277 two species, being 8.1 ± 0.1 in halibut and 7.2 ± 0.1 in cod when assayed at 37 ± 1°C. At pH 7,

278 the activity was reduced to 86 and 99.5% of the maximum in halibut and cod respectively.

279

280 K_M is the substrate concentration at half maximum enzyme activity and an estimate of the

281 dissociation constant of the enzyme and the utilized substrate under the given condition. The

282 K_M for AP was 0.92 and 1.45 mM in halibut and cod, respectively. Further, the K_M of LAP

283 and Leu-ala were approximately 0.2 and 0.1 mM, respectively, in both species (Table 1).

284

285 *3.2. Growth*

286

287   The halibut larvae were 12.1±0.6 mm in length and 5.4±0.6 mg in wet weight at first feeding

288   and experimental start (Fig. 1a-b). At the last sampling, the larvae had been transferred to

289   weaning tanks and the sample was separated in two based on size and developmental stage

290   (See materials and methods). The most developed group had achieved juvenile pigmentation

291   pattern. The lengths of the larvae were 25.9±2.8 and 31.1±1.8 mm in the less and more

292   developed group, respectively, and the corresponding wet weights were 237.0±68.1 and

293   442.0±74.7 mg. The growth was exponential and is approximately described by the models

294    $SL=11.15*e^{(0.011*dpff)}$ (R^2=79.2; $\underline{P}$<0.0001; 1-71 dpff) and Weight=$3.35*e^{(0.060*dpff)}$ (R^2=94.3;

295    $\underline{P}$<0.0001; 1-71 dpff). Calculated from wet weight and using both groups of halibut at the last

296   sampling, SGR were on average 5.0-5.9 % day^{-1}.

297

298   The cod larvae were 4.0±0.7 mm at 7 dpff and 30.0±4.8 mm at 72 dpff (Fig. 1a). The growth

299   profile until 51 dpff is given by the model $SL=3.39*e^{(0.019*dpff)}$ (R^2=81.8; $\underline{P}$<0.0001).

300   Thereafter, the growth rate increased and was better expressed by the model:

301    $SL=0.48*e^{(0.057*dpff)}$ (R^2=88.9; $\underline{P}$<0.0001).

302

303   *3.3. Ontogenetic development of enzymes*

304

305   Enzyme activities in halibut and cod, when expressed per larvae, increased gently from first

306   feeding to about 30 dpff ($\underline{P}$<0.02; Fig. 2a-b). In the second half of the experimental period of

307   halibut, Leu-ala proceeded with a gradual eightfold increase in individual activity until the

308   end of the experiment (Leu-ala= -780.1+28.5*dpff (R^2=95.5); 29-71 dpff; $\underline{P}$<0.0001), while

309   AP and LAP increased sharply 19-23-fold from 34 to 52 dpff (AP=68.8+2.059*dpff

310   (R^2=86.4); LAP=50.8+1.55*dpff (R^2=94.3); $\underline{P}$<0.0003). Thereafter the activities of AP and

311   LAP remained statistically unchanged, but varied greatly between replicates. The sharp

312 increases in activities of AP and LAP in halibut corresponded to the period of highest SGR

313 during the experiment, being 10.6% day^{-1} from 29 to 52 dpff. Before 29 dpff the growth rate

314 was 2.6% day^{-1}, while after 52 dpff it was 2.8 – 5.3% day^{-1} calculated on basis of the least and

315 most developed larval group at 78 dpff, respectively. A different picture was seen in cod,

316 where the individual activities of all three enzymes increased exponentially from 29 dpff and

317 onwards (AP=0.031*e$^{(0.12*dpff)}$ (R^2=98.0); LAP=0.012*e$^{(0.11*dpff)}$ (R^2=96.6); Leu-

318 ala=0.27*e$^{(0.093*dpff)}$ (R^2=96.9); 29-72 dpff; P<0.0001).

319

320 The specific activities of AP and LAP in halibut changed almost similarly during the

321 experimental period (Fig. 3a-b). Their activities were low and unchanged from first-feeding

322 until 29 dpff and then increased approximately fivefold from 29 to 52 dpff (AP= -

323 1075.7+44.8*dpff (R^2=85.9); LAP=-795.1+35.0*dpff (R^2=92.9); P<0.0001). Thereafter the

324 activity of AP remained at the same level, except for an elevated level in the most developed

325 larvae at 78 dpff (P=0.0002). The specific activity of LAP was high and unchanged from 52

326 to 58 dpff, and thereafter it was reduced to about 50% (P=0.0002). Similarly as for AP, the

327 activity of LAP was also elevated in the most developed larvae at 78 dpff compared to the

328 smaller larvae of the same age and the two previous samplings (P=0.005). The specific

329 activity of Leu-ala in halibut increased 11-fold from first feeding to 34 dpff (Leu-

330 ala=51.6*e$^{(0.073*dpff)}$ (R^2=91.5); P<0.0001; Fig. 3c). At 52 dpff, the activity was reduced to

331 approximately 65% of the activity at 34 dpff (P=0.006) and then remained at a steady level

332 until the end of the experiment.

333

334 In cod, the specific activities of AP and LAP remained unchanged during the first 29 dpff

335 (Fig. 4a-b). From 29 to 37 dpff an approximately threefold increase in activity of AP (P=0.02)

336 was present. The activity remained unchanged until 59 dpff, and thereafter it declined to

337   approximately half of its activity at 37-59 dpff, although not significantly ($\underline{P}$=0.06). LAP

338   showed on average twofold higher activity at 37 dpff than at all other sample dates ($\underline{P}$<0.03

339   except for $\underline{P}$=0.15 at 43 dpff). The elevated activities of AP and LAP at 37 dpff corresponded

340   with a change in diet from rotifers to Artemia. Random variation in specific activity of Leu-

341   ala in cod was found during the first 29 dpff (Fig. 4c). Similarly to AP and LAP, maximum

342   specific activity was present at 37 dpff ($\underline{P}$<0.003). For AP and LAP, it was confirmed by

343   reanalyses of whole larvae homogenates from 37 to 51 dpff that the changes in activities from

344   29 to 37 dpff were not due to the methodological change from analysis of whole larvae to

345   analysis of gut segments.

346

347   The activities of AP and LAP were determined both in the homogenates of whole larvae or

348   gut segments and in the bbm extracts. The activities in the bbm extracts in percent of the total

349   individual activity measured in the homogenates were calculated and are shown in figures 5a-

350   b. In halibut, these calculations described a quite similar pattern as the specific activities,

351   while a different picture was seen in cod. In halibut, the activities of the bbm bound enzymes

352   increased from constituting 2% at 34 dpff and earlier, to approximately 15% of the total

353   individual activity at 43 dpff ($\underline{P}$<0.0003). Thereafter the ratio of membrane bound to total

354   activity remained statistically unchanged. Low percentages of membrane bound activities of

355   AP and LAP were also present in the beginning of the experiment with cod, before marked

356   increases occurred. In contrast to the result in halibut, increasing percentages of the bbm

357   bound activities of LAP and AP were found at different ages. Bbm bound activity of AP

358   increased from constituting 5% of total activity at 29 dpff to 22% at 51 dpff (AP= -

359   16.4+0.76*dpff (R^2=81.1); $\underline{P}$<0.0001), while bbm bound activity of LAP increased

360   exponentially from 5% at 37 dpff to 31% at 72 dpff (LAP=0.70*e$^{(0.051*dpff)}$ (R^2=90.2);

361    $\underline{P}$<0.0001).

362

363 **4. Discussion**

364

365 *4.1. Optimisation of the enzyme assay used*

366 Prior to the ontogenetic study, the enzyme assays were optimised for substrate concentration,

367 temperature and pH. The two latter parameters were not determined for Leu-ala due the

368 complexity of the assay. Further optimisation could have included testing the effects of ionic

369 strength and the concentration of all components in the assay mixture since these likely also

370 will impact on enzyme activity (Tipton, 2002), but the specified optimisation was considered

371 sufficient for our purposes. The determination of optimal conditions during assay had two

372 purposes. Firstly, this ensures a linear increase in product release during the assay interval,

373 which is demanded for obtaining valid quantitative data (Tipton, 2002). In the present study,

374 the low temperature optimum of AP, especially in halibut, was important information for

375 avoiding enzyme denaturation during assay run. Assay temperature for AP in halibut was

376 therefore set to 30°C, in agreement with the optimum conditions determined. Assay

377 temperature for AP from cod was set to 37ºC, although a little lower optimum temperature

378 was found.

379

380

381 The second purpose for assay optimisation was to maximise the enzyme activity potential. At

382 determined optimums of pH and temperature, the specific activities were up to twice as high

383 as the activities at the conditions that would have been used if the optimisation study had not

384 been performed (37ºC and pH 9.8 and 7 for AP and LAP, respectively). The optimum pH was

385 specified as assay condition for each enzyme and species in the ontogenetic study, whereas

386 this was not always the case with the optimum temperatures. The optimum temperature for

387   LAP was very high and difficult to keep constant during assay. The assay temperature was

388   therefore set to 37ºC for both species.

389

390   The analysed K_M values were fairly similar in the two species, although AP in halibut seems

391   to have higher affinity for the substrate than AP in cod. The recommended range of substrate

392   concentrations for estimating K_M is from 0.5 to 10 times the K_M (Henderson, 1992).This

393   substrate range was covered in the present study, except for in the Leu-ala assay where the

394   lowest substrate concentration was 0.6-0.8 times the K_M. The assay substrate concentrations

395   used were from 4.8 to 30 times higher than the estimated K_M values, ensuring that substrate

396   concentration was in access during assay.

397

398   *4.2. Ontogenetic development*

399

400   The growth of halibut appeared to be slow in periods (i.e. 1-29 and 52-71 dpff). However, the

401   size distribution of the larvae in the samples from 52 to 71 dpff was probably not

402   representative for the size distribution in the rearing tank. The gentle break in the growth

403   profile at 52 dpff, arising at approximately 22 mm SL, seems to coincide with the time of

404   metamorphic climax when the larvae begin to settle to the bottom (Sæle et al., 2004). Since

405   samples were collected from the upper half of a large rearing tank, the largest larvae were

406   likely not represented in the samples after 52 dpff. The significantly larger size of the larvae

407   in the sample at 78 dpff that included the largest individuals also supports this. In cod, the

408   growth appeared to be slow in the beginning of the experiment, as compared with other

409   studies (Baskerville-Bridges and Kling, 2000; Callan et al., 2003; van der Meeren and

410   Ivannikov, 2006), but the growth in body length markedly improved after 50-60 dpff. During

411   the experimental periods with both species, the rearing outcome was considered normal with

412   no particular problem that altered the larval quality. Thus, the larvae used in the studies were

413   considered to be representative for their respective species.

414

415   The change in digestion from the larval to the adult mode was expected indicated by

416   concurrently occurring decrease in Leu-ala specific activity and increase in AP and LAP

417   specific activities (Zambonino Infante and Cahu, 2001). The background for using Leu-ala as

418   a marker for decreasing larval mode of digestion is its proposed involvement in the terminal

419   steps of intracellular digestion of pinocytically absorbed protein (Henning, 1987; Zambonino

420   Infante and Cahu, 2001). In halibut, a marked decrease in pinocytosis is found at the end of

421   metamorphosis (Luizi et al., 1999), which was passed at 78 dpff in the present study.

422   However, Leu-ala failed to show the expected decrease in both halibut and cod. Declining

423   specific activity of Leu-ala is found in Senegalese sole (Solea senegalensis) and sea bass

424   (Dicentrarchus labrax) between approximately 20 and 30 dph (Ribeiro et al., 1999;

425   Zambonino Infante and Cahu, 2001). Thus, the present study opposes with results in other

426   species.

427

428   In cod, Leu-ala appears to show an overall decrease during the second part of the experiment,

429   but the large variance in the data makes it difficult to conclude on this point. Likely, the

430   sampling of cod after the morning feed distribution has had a major impact on the specific

431   activity of Leu-ala, and also the diet changes during the experiment may have altered the

432   results. Therefore, Leu-ala appears to give little information of the digestive processes in cod.

433

434   In halibut, the inclusion of more organs in the enzyme extract may have camouflaged a

435   potential decrease in cytosolic Leu-ala activity in the hindgut, which is the part of the intestine

436   where intracellular protein digestion takes place. Dipeptidases have a general role in cellular

437   metabolism of protein and are widely distributed in different body tissues (Kim et al., 1972).

438   Moreover, along with their cytosolic presence in the enterocytes, they are also anchored in the

439   bbm, although only a minor portion of Leu-ala is thought to be present in the bbm in the

440   intestine of the rat (10-30 %; Kim et al., 1972; Nicholson and Kim, 1975). This portion may,

441   however, be underestimated due to difficulties in separating the bbm and cytosolic fractions

442   (Ugolev and De Laey, 1973; Kim et al., 1972). Thus, besides its proposed role in larval mode

443   of digestion, Leu- ala may also participate in the adult mode, both by intracellular and surface

444   digestion. Accordingly, Leu-ala activity assayed in the intestinal segment of the larvae may

445   not be a suitable marker for larval mode of digestion.

446

447   Whereas Leu-ala failed to show the expected ontogenetic profile, abrupt increases in AP and

448   LAP specific activities were noted between 30 and 40-50 dpff in both halibut and cod. In cod,

449   the increases concurred with the shift from analysing enzyme activities in whole larvae to

450   analysing the activities in the intestinal segment of the larvae, but reanalyses of homogenates

451   of whole larvae and intestinal segment of the larvae from 37 to 51 dpff confirmed that AP and

452   LAP were mainly situated in the intestinal segment of the larvae (Results not shown). Similar

453   increases in specific activities of AP and LAP are found in other species (Ma et al., 2005;

454   Ribeiro et al., 1999; Zambonino Infante and Cahu, 2001). Thus, the results of AP and LAP in

455   halibut and cod agree with findings in other species.

456

457   In the present study, the activities of bbm-bound AP and LAP were expressed as specific

458   activity, as individual activity and as activity in percent of total activity of the respective

459   enzyme in the intestinal segment of the larvae or whole larvae. By expressing the activity in

460   several ways, the real trend of the enzyme might be better understood as the numerators

461   (soluble protein and total intestinal activity of the enzyme per larva) might change over time

462   and hence influence the profiles. This is particularly true when more organs than the gut are

463   included in the enzyme extracts, as is the case in the present study.

464

465   With exception of LAP in cod, the expression of the activities of AP and LAP as percentage

466   of the total activity of the respective enzyme in the intestinal segment of the larvae supported

467   the ontogenetic profiles in specific activity. The fraction of the enzymes recovered in the bbm

468   increased from approximately 5% to 15-20% and coincided with the increases in specific

469   activities, indicating an increased importance of surface digestion from the time when the

470   increases had occurred. In other studies, a marked increase in the activity ratios of bbm-

471   enzymes to Leu-ala have been used to describe the change from larval to adult mode of

472   digestion (Cahu et al., 1999; Ma et al., 2005). In the present study, such ratios showed

473   approximately similar profiles (Results not shown) as the activity ratios of bbm-bound AP

474   and LAP in the intestinal segment of the larvae, in both halibut and cod. Thus, the activity

475   ratio profiles showed in the present study seem to reflect a similar developmental process as

476   the ratios of bbm-enzyme to Leu-ala activity.

477

478   The specific activity of LAP decreased significantly after 50 dpff in both species. In the

479   profiles of the activity ratio of bbm-bound LAP activity to total LAP activity in the intestinal

480   segment of the larvae, this decrease was less marked in halibut and not present in cod. Thus,

481   the decreases in LAP specific activity were probably related to physiological changes in the

482   soluble protein in the enzyme extract.

483

484   The abrupt elevations in bbm-enzymes may relate to structural changes of the intestinal

485   mucosa. Coincident with increased specific activities of bbm enzymes, increased folding of

486   the intestinal mucosa is indicated in other species (Zambonino Infante and Cahu, 2001). The

487   achievement of this highly folded mucosa, which characterises a mature and healthy intestine,

488   both increase the intestinal surface and may also indicate that the process of cell proliferation

489   and differentiation in the bases and tips of the folds, respectively, become more efficient.

490   These points may explain the abrupt increase in specific activities of bbm-bound enzymes,

491   which thus can be noted as maturational increases. In cod, increased folding of the intestinal

492   mucosa was found in 0.17 mg dry weight larvae (Kjørsvik et al., 1991). When carrying out a

493   weight to SL conversion based on the data to Finn et al. (2002), this larval size corresponds to

494   approximately 7 mm SL, which was about the size of the larvae when AP and LAP specific

495   activities increased in the present study. In halibut, increased folding of the intestinal mucosa

496   is found during the course of metamorphosis (Luizi et al., 1999), defined to be from

497   approximately 22 to 25 mm SL (Sæle et al., 2004). Thus, folding of the mucosa appears to

498   occur after the elevated bbm enzyme activities at 18-22 mm SL found in the present study.

499   The lack of coincidental occurrence of these two events in halibut might be real or due to

500   difficulties in matching the data from two different studies that used quite different methods

501   to study developmental progress. Furthermore, different rearing conditions may have caused

502   slight variations in development. Even though the coincidences between the increases in bbm-

503   enzymes and the achievement of increased folding of the intestinal mucosa may be uncertain,

504   the increases in AP and LAP specific activities in halibut and cod can be considered to

505   represent maturational increases, in agreement with the ontogenetic profiles in other species

506   (Ribeiro et al, 1999; Zambonino Infante and Cahu, 2001; Henning et al., 1994).

507

508   As compared to AP, LAP in cod showed a less marked increase in specific activity and a

509   delayed increase in ratio of bbm-bound activity to total activity in the intestinal segment of

510   the larvae. This pattern differed from the results in halibut where the enzymes showed similar

511   ontogenetic profiles. The deviating LAP profiles in cod may relate to the diet change from

512 rotifers to <u>Artemia</u> between 35 and 40 dpff. However, the 5 days co-feeding with both feed

513 organisms means that the diet change was gradual. Alternatively, the deviating LAP profiles

514 can be due to the feeding state of cod at sampling, although LAP activity was noted not to be

515 affected by feeding in a semi-quantitative study (Cousin et al., 1987). As a third possibility,

516 LAP may reach a maturational activity level at a later stage than AP in cod. Also in other

517 species, a less steep increase in the activity of LAP than of AP is described (<u>Pseudoscriaena</u>

518 <u>crocea</u>; Ma et al., 2005). For attaining a mature activity level, LAP may depend on surplus

519 availability of oligopeptides as substrate in the gut lumen and thus on the diet composition

520 and on the activities of gastric and pancreatic enzymes which act on the dietary protein prior

521 to LAP (Reisenauer and Castillo, 1994). In cod, the development of the stomach appears to be

522 delayed as compared to in some other fish species (Pedersen and Falk-Petersen, 1992).

523 Pedersen and Falk-Petersen (1992) found that the stomach started to develop at 15 mm SL in

524 cod, which in the present study correspond to the size of the larvae at 60-65 dpff and to the

525 time when the activity ratio of bbm-bound LAP to total LAP in the intestinal segment of the

526 larvae increased. Thus, the less clear achievement of mature activity of LAP than of AP in

527 cod may relate to the developmental process of the stomach.

528

529 The significantly higher specific activities of AP and LAP in the most developed halibut

530 larvae at 78 dpff, compared to the other larvae group of same age, may relate to a more

531 completely established gastric digestion in the former group. The higher enzyme activities in

532 these larvae suggest that they were able to more efficiently digest the formulated diet that was

533 offered from 74 dpff and onwards. Luizi et al. (1999) found that the stomach was developed

534 by the end of metamorphosis, and Stoss et al. (2004) report that gastric digestion is considered

535 developed at 80 dpff in halibut. Thus, the stomach was probably developed in both groups

536 sampled at 78 dpff, but the stomach in those individuals that had achieved a juvenile

537 appearance was likely more fully functional.

538

539 Marked increases in activities of bbm enzymes are supposed to indicate the time when the

540 larvae have acquired a more adult-like intestinal functionality and, thus, improved ability to

541 digest formulated diets. Comparing with feeding experiments, successful weaning of halibut

542 has been reported from 70 to 120 mg wet weight when Artemia supplementation was given

543 during the first week of weaning (Næss et al., 2001; Kvåle et al., 2002). This agrees with the

544 time of intestinal maturation found in the present study. After 1-2 weeks co-feeding with

545 rotifers, cod has been completely weaned at 22 dah and 8-8.5 mm SL with good results

546 (Baskerville-Bridges and Kling, 2000; Callan et al., 2003). This is earlier than the time of

547 intestinal maturation found in the present study, according to age, but fairly concomitant

548 according the larval size. In all these studies (Baskerville-Bridges and Kling, 2000; Næss et

549 al., 2001; Kvåle et al., 2002; Callan et al., 2003), weaning was considered early as compared

550 to what possible with the diet used. Thus, the comparisons indicate that intestinal maturation

551 might be a significant factor for success in weaning. However, good weaning results depend

552 on a diet that fulfils the larval requirement. Thus, using diet that takes into account the

553 digestive features of the fish larvae before acquisition of a mature intestine, weaning may

554 succeed at an earlier developmental stage.

555

556 In conclusion, marked increases in activities of bbm-bound AP and LAP were found in

557 halibut and cod, indicating that a mature intestine was reached at 40-50 dpff in both species,

558 corresponding to 50-114 mg wet weight in halibut and 7-9 mm SL in cod. However,

559 diverging activity profiles of LAP and AP made a less clear picture of intestinal maturation in

560 cod than in halibut, and demonstrate the complexity of the maturational processes of the

561   intestines. Comparing with early weaning studies, intestinal maturation might be a significant

562   factor for successful weaning, but more studies are needed to confirm this. In the present

563   study, Leu-ala failed to indicate decline in the larval mode of digestion in both halibut and

564   cod.

565

566   **Acknowledgements**

567

572

573   **References**

574

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682   **Caption to figures**

683   Fig. 1. Growth measured as a) standard length (SL; mm) and b) wet weight (mg) of Atlantic

684   halibut (1-78 dpff) and Atlantic cod (0-72 dpff; only SL). Two samples of halibut were

685   collected at 78 dpff, differentiated on basis of size and development (See materials and

686   methods). The most developed group is plotted at 80 dpff for visualisation purposes. Data are

687   means±sd (n=10-50), and different letters indicate statistically significant differences. Lower

688   case letters describe halibut and upper case letters cod.

689

690   Fig. 2. Enzyme activity per larva (mU(larva)$^{-1}$) in a) Atlantic halibut (1-78 dpff) and b)

691   Atlantic cod (0-72 dpff). Two samples of halibut were collected at 78 dpff differentiated on

692   size and development (See materials and methods). The most developed group is plotted on

693   80 dpff for visualisation purposes. The portions of larvae used in the enzyme extracts are

694   whole larvae (cod, 0-29 dpff) and the intestinal segment of the larvae (cod, 37-72 dpff;

695   halibut, 1-78 dpff). The enzymes are brush border membrane alkaline phosphatase (AP),

696   brush border membrane leucine aminopeptidase N (LAP) and the leucine-alanine peptidase

697   (Leu-ala). Data are means±sd (n=3), and different letters indicate statistically significant

698   differences. Upper case letters describe AP, lower case letters LAP and italic letters Leu-ala.

699   In a) the left axis denotes AP and LAP and the right axis denotes Leu-ala.

700

701   Fig. 3. Specific enzyme activity (mU (mg protein^{-1}) of a) brush border membrane alkaline

702   phosphatase (AP), b) brush border membrane leucine aminopeptidase (LAP) and c) leucine-

703   alanine peptidase (Leu–ala) in Atlantic halibut from 1 to 78 dpff. Two samples were collected

704   at 78 dpff differentiated on basis of size and development (See materials and methods). The

705   most developed group is plotted on 80 dpff for visualisation purposes. The portion of larvae

706 used in the enzyme extracts is the intestinal segment. Data are means±sd (n=3), and different

707 letters indicate statistically significant differences.

708

709 Fig. 4. Specific enzyme activity (mU (mg protein)$^{-1}$) of a) brush border membrane alkaline

710 phosphatase (AP), b) brush border membrane leucine aminopeptidase (LAP) and c) leucine-

711 alanine peptidase (Leu–ala) in Atlantic cod from 0 to 72 dpff. The portions of larvae used in

712 the enzyme extracts are whole larvae (0-29 dpff) and the intestinal segment (37-72 dpff). Data

713 are means±sd (n=3), and different letters indicate statistically significant differences.

714

715 Fig. 5. Activity of the brush border membrane bound alkaline phosphatase (AP) and leucine

716 aminopeptidase N (LAP) in percent of total activity of the respective enzyme in the

717 homogenate of whole larva or gut segment in a) Atlantic halibut (1-78 dpff) and b) Atlantic

718 cod (0-72 dpff). Two samples of halibut were collected at 78 dpff differentiated on basis of

719 size and development (See materials and methods). The most developed group is plotted on

720 80 dpff for visualisation purposes. The portions of larvae used in the enzyme extracts are

721 whole larvae (cod, 0-29 dpff) and the intestinal segment (cod, 37-72 dpff; halibut, 1-78 dpff).

722 Data are means±sd (n=3), and different letters indicate statistically significant differences.

723 Upper case letters describe AP and lower case letters LAP.

724 **Table**

725

726 Table 1. Temperatures and pH optimum values and Michaelis constants (K_M) for intestinal

727 alkaline phosphatase (AP), leucine aminopeptidase N (LAP) and leucine-alanine peptidase

728 (Leu-ala) in Atlantic halibut and Atlantic cod larvae. K_M was determined by direct linear plots

729 and the analysed ranges of substrate concentrations compared to K_M are noted.

Enzyme	Species	Temperature optimum[a] (°C)	pH optimum[b]	Substrate range (x K_M)	K_M (mM)
AP[c]	Halibut	30	10.3	0.2-16	0.92
	Cod	34	10.1	0.2-10	1.45
LAP[c]	Halibut	42	8.1	0.2-14	0.17
	Cod	47	7.2	0.5-10	0.22
Leu-ala[c]	Halibut	nd[d]	nd	0.6-14	0.11
	Cod	nd	nd	0.8-32	0.08

730 a) Assay pH values were 9.8 for AP and 7.0 for LAP. b) Assay temperature was 30°C for AP

731 in halibut, elsewhere 37°C. c) Substrates are *p*-nitro phenyl phosphate for AP, Leucine-*p*-

732 nitroanilide for LAP and leucylalanine for Leu-ala. d) nd = not determined.

733
734   Figure 1a

735
736   Figure 1b
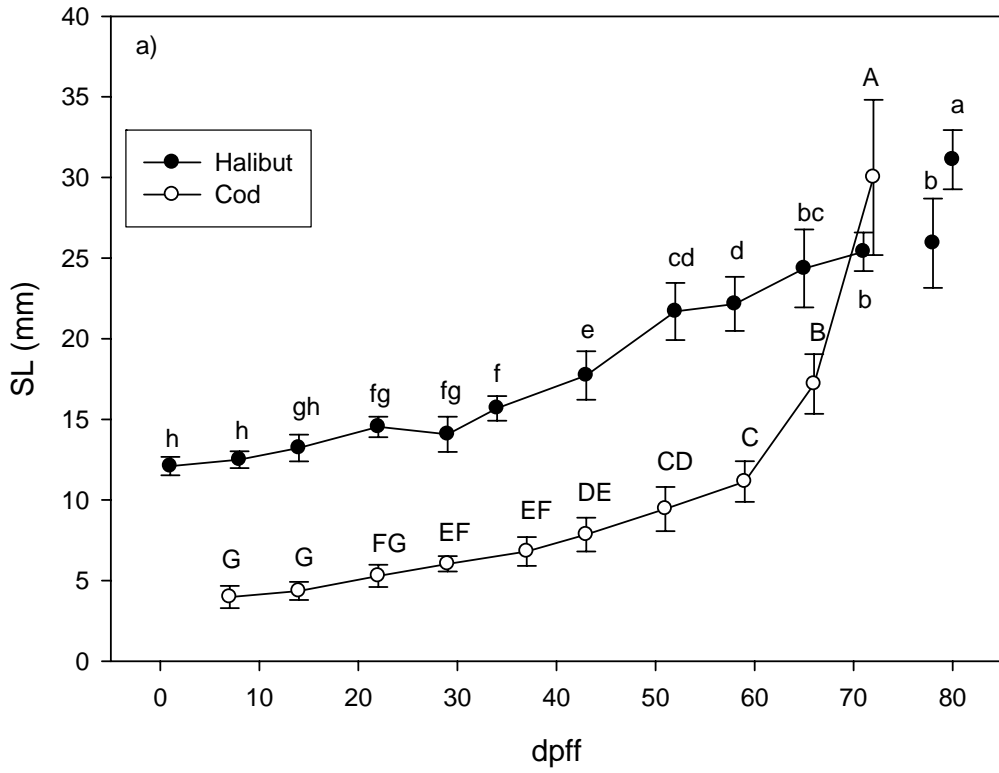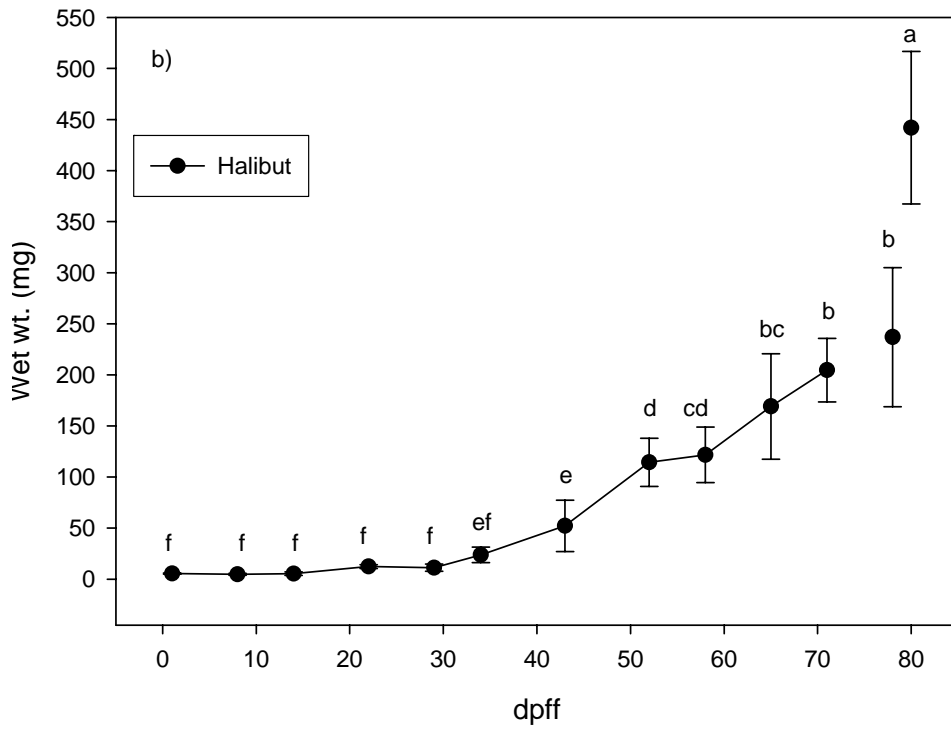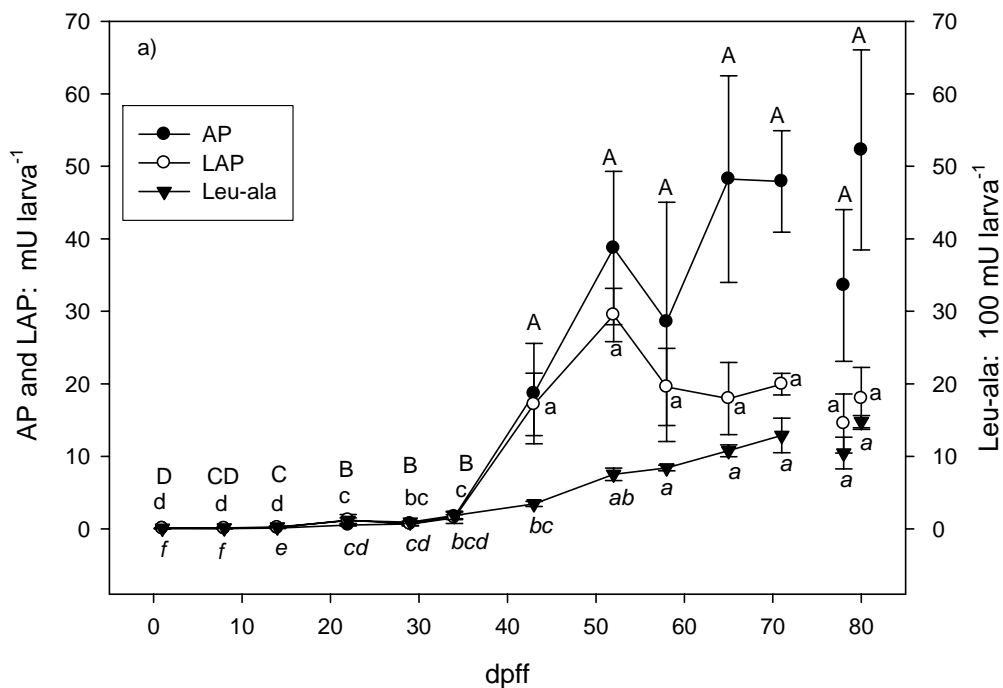
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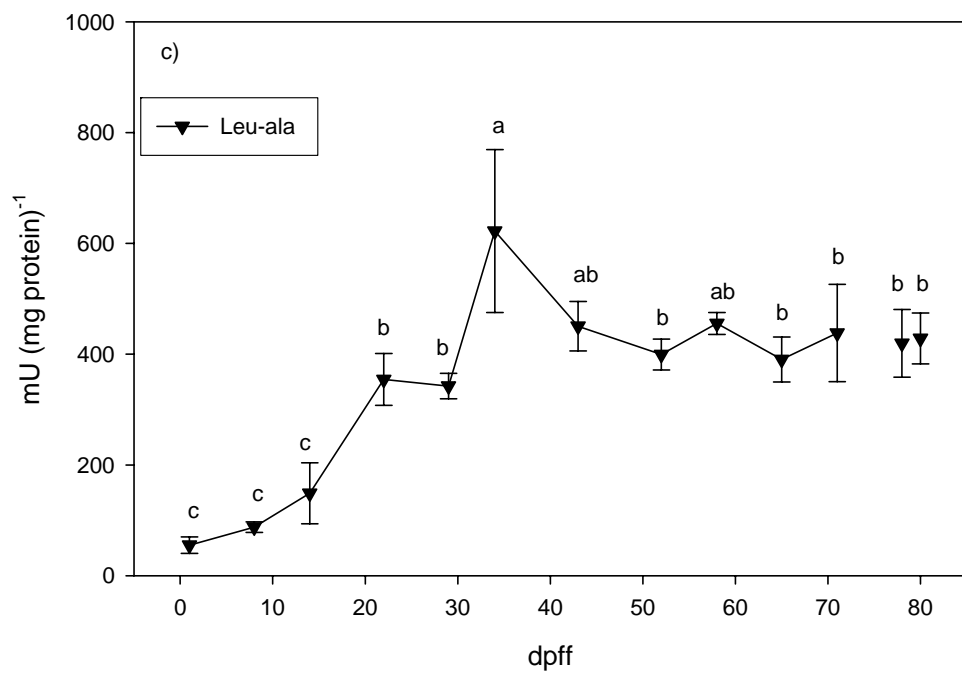738   Figure 2a
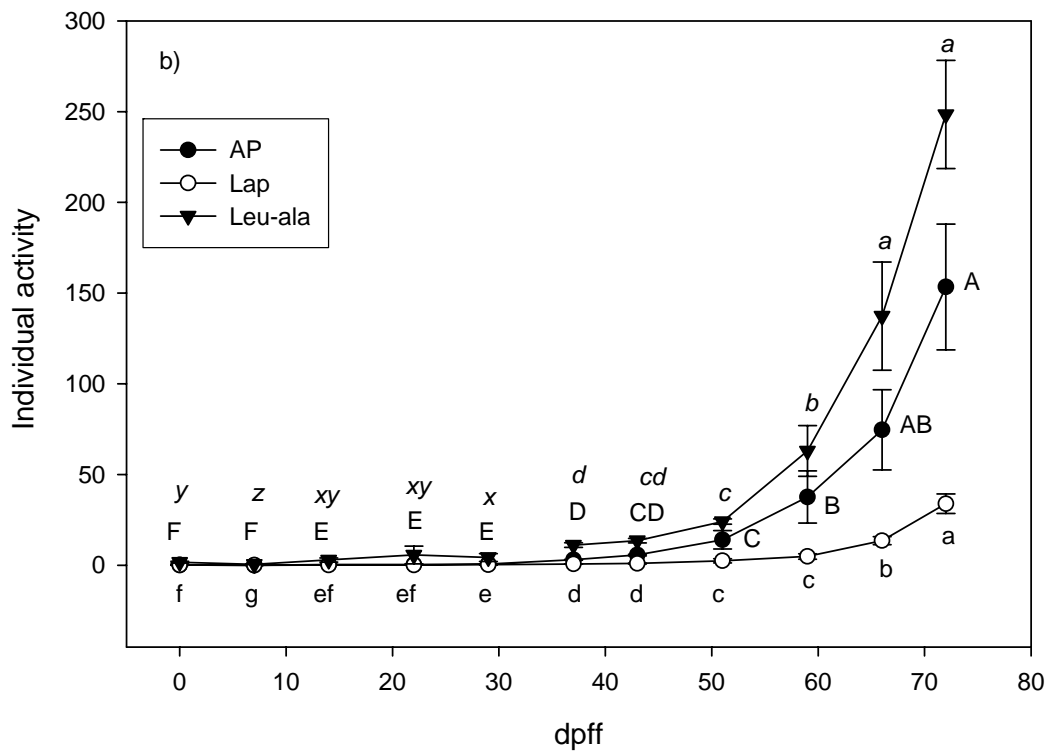
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740   Figure 2b
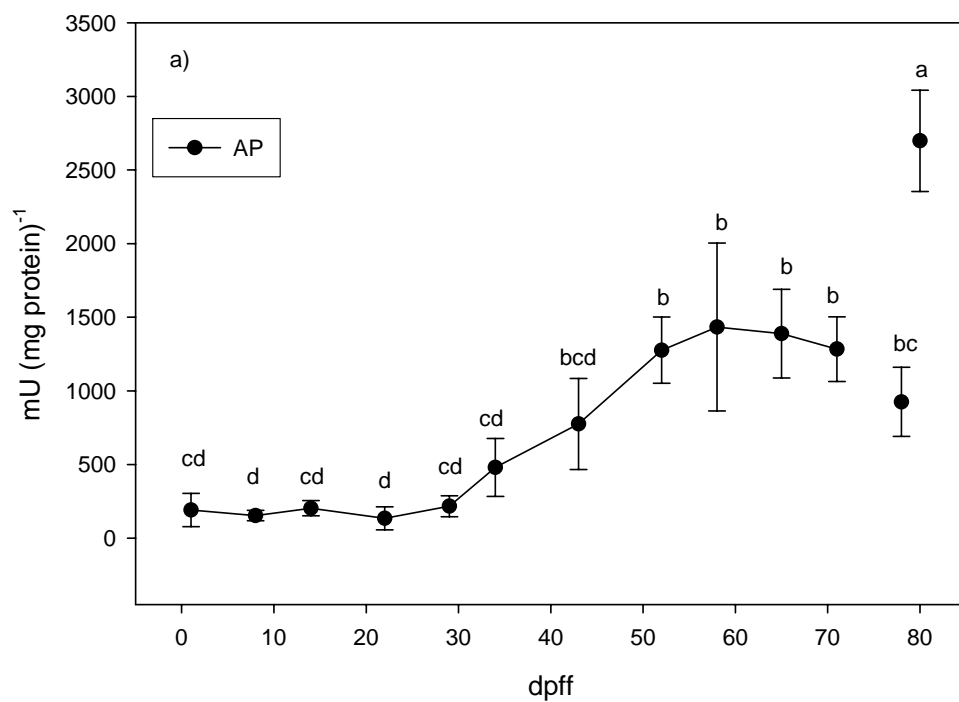
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742   Figure 3a
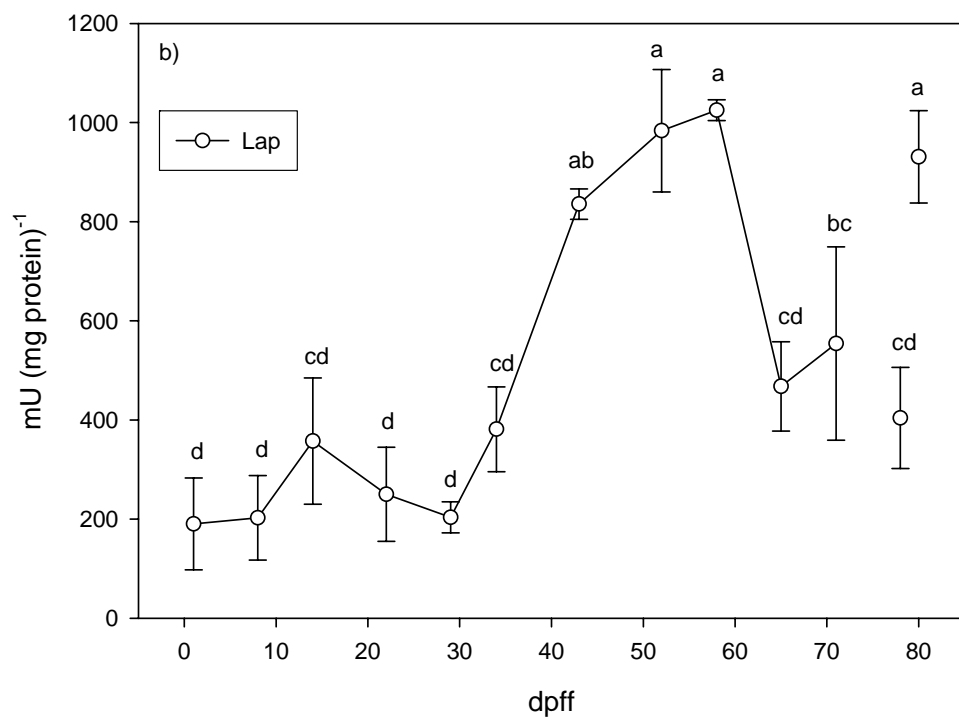
743
744   Figure 3b

745
746   Figure 3c
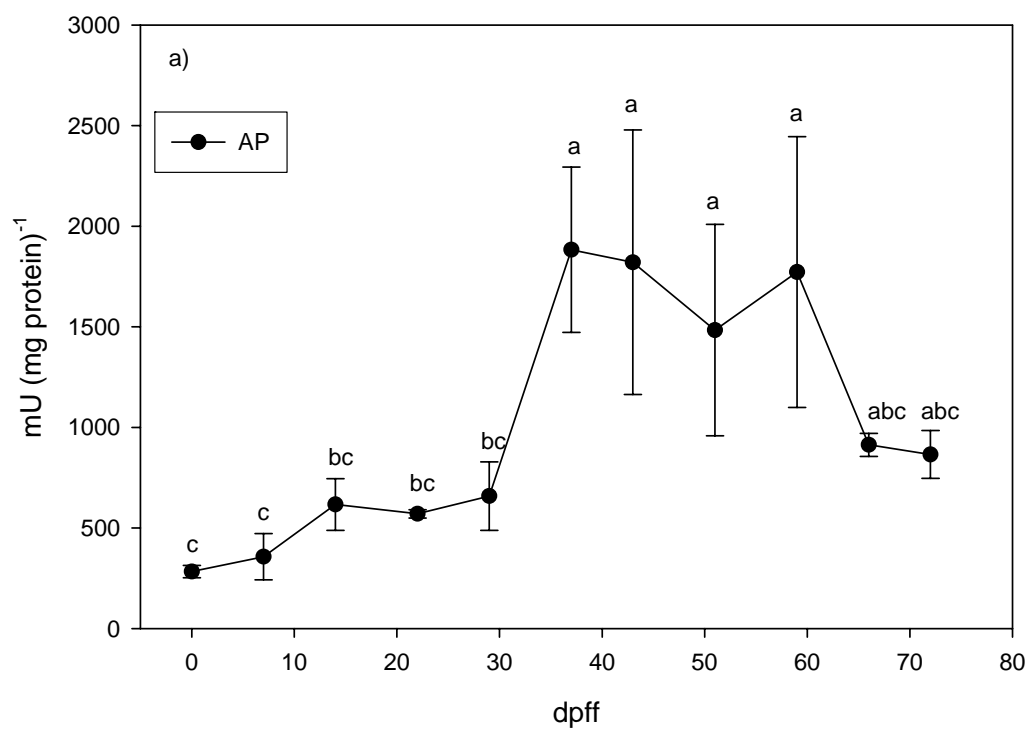
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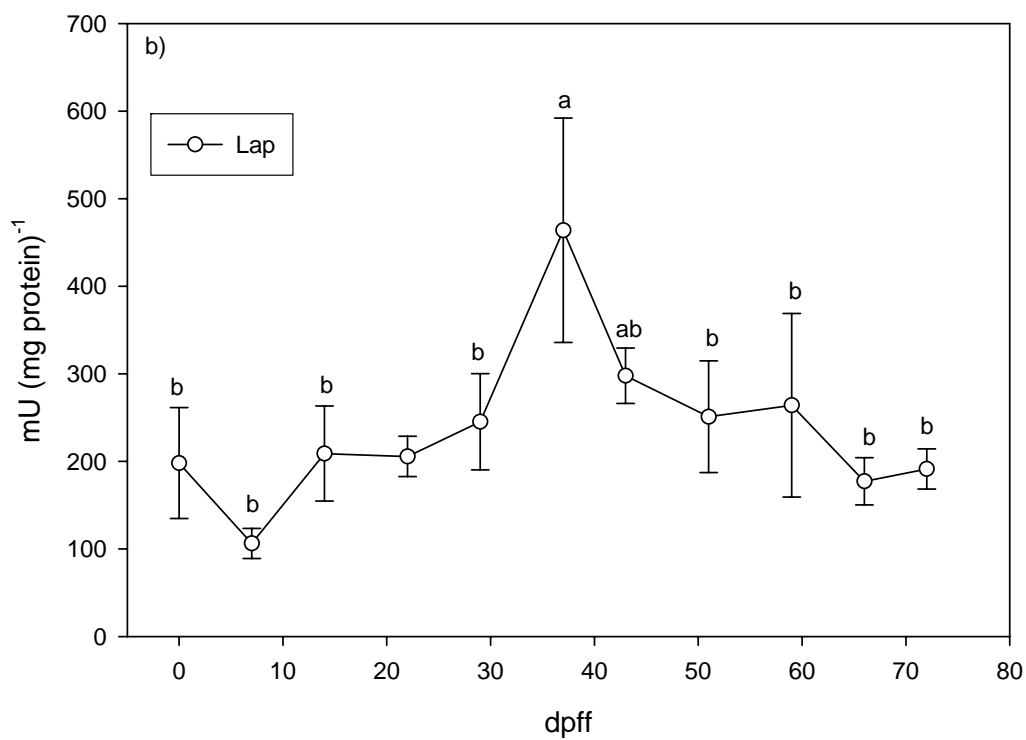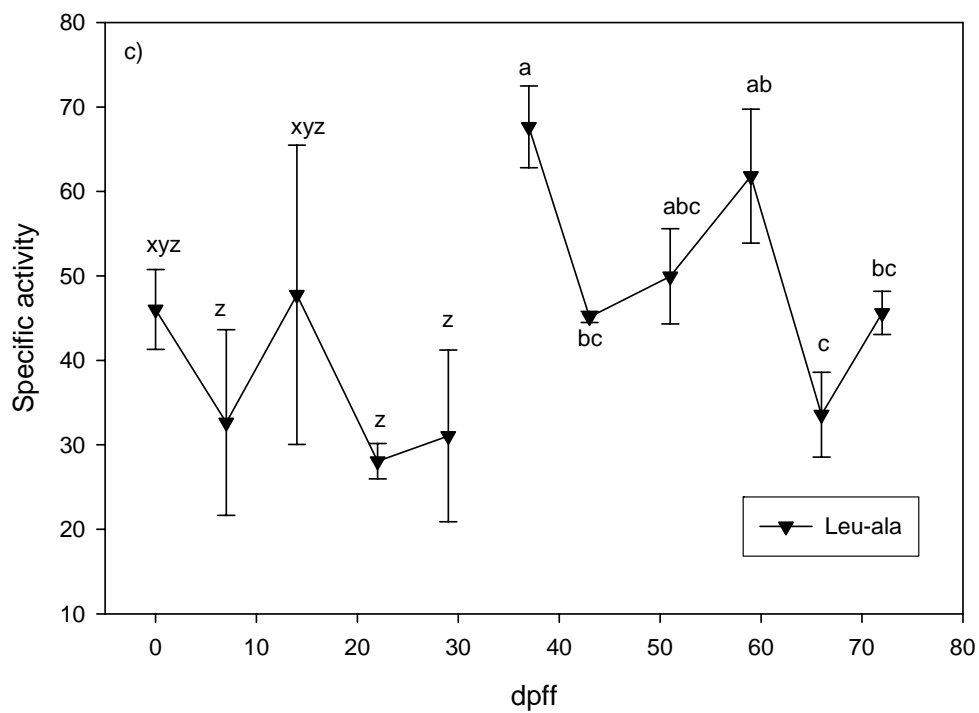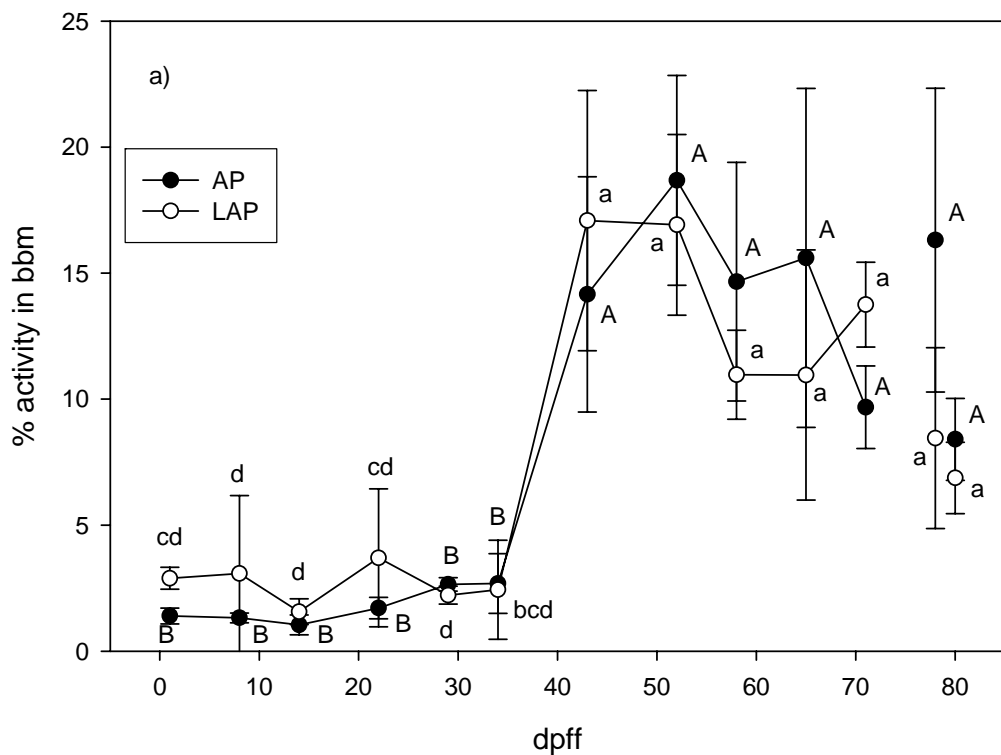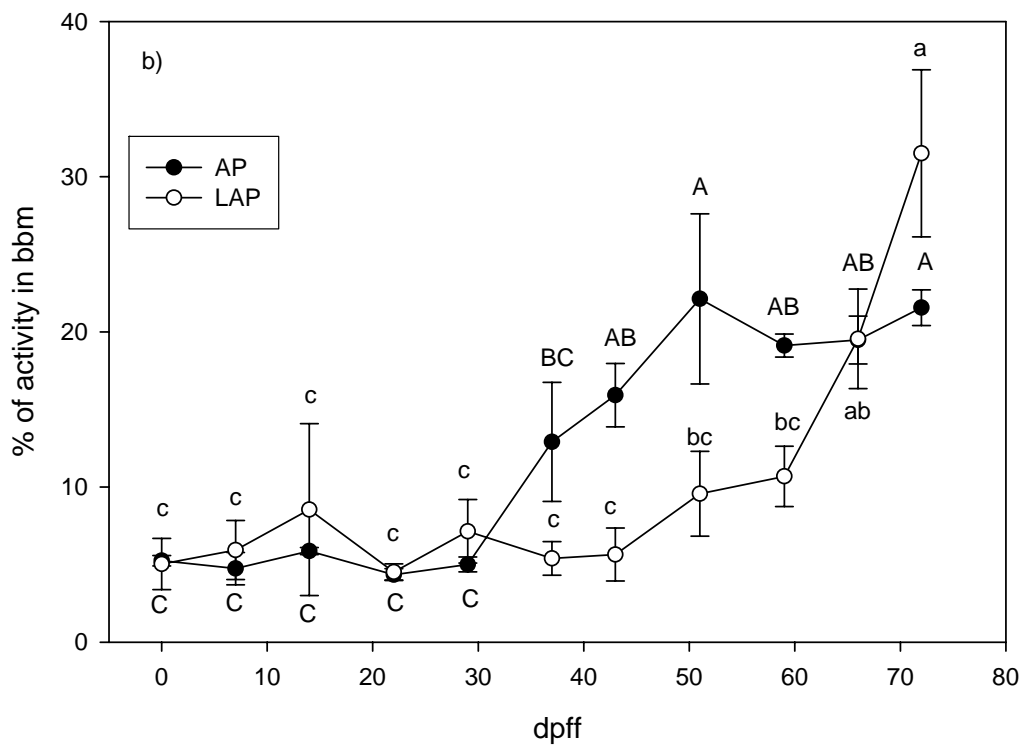748   Figure 4a

749
750   Figure 4b

751
752   Figure 4c

753
754   Figure 5a

755
756   Figure 5b