Comparative ontogeny of the digestive tract of *Oncorhynchus mykiss* ♀ x *Salmo trutta caspius* ♂ triploid hybrids to their parental species

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Running Title: Ontogeny of digestive tract

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ABSTRACT

The ontogeny of the gastrointestinal tract of the hybrid between female rainbow trout, RT (*Oncorhynchus mykiss*) and male Caspian brown trout, CBT (*Salmo trutta caspius*) was compared to the parental species. Larvae were collected for histology and enzymatic assays (amylase, lipase and trypsin) at 3, 6, 9, 12, 15, 18, 21, 26, 31, 35, 40 and 45 days post hatch (dph). The development of the gastrointestinal tract (GI-tract) and the onset of digestive enzyme production was variable between groups. The GI-tract post hatch was a relatively simple tubular structure and a rudimentary esophagus was differentiated from other regions at 3 dph in all studied groups. The pyloric caeca and the U-shaped stomach were clearly visible at 26, 35 and 40 dph in RT, triploid hybrid and CBT, respectively. An abrupt increase in trypsin activity at 31, 35 and 45 dph, was identified in CBT, RT and the triploid hybrid, respectively. The increasing activity of trypsin and decreasing activity of lipase during larval development suggests that the CBT, RT and triploid hybrid rely more on dietary proteins than lipids with increasing age. The hybrid grew better and had a faster GI-tract development than CBT, while RT performed best overall.

KEYWORDS: Gastrointestinal development; Digestive enzymes; Larval development; Salmonid; Hybridization

1. INTRODUCTION

Hybridization or the crossing of individuals from the same species (crossbreeding) or different species (interspecific) offers the possibility of improving traits that benefit aquaculture. Hybridization in salmonids has been used to improve disease resistance, growth rates, manipulate sex ratios and to produce sterile fish (Bartley et al., 2000). For example, *Salmo trutta* x *Salvelinus fontinalis* hybrids are sterile (Scheerer & Thorgaard, 1983). The hybrids of *Salvelinus namaycush* x *S. fontinalis* (maintained at low pH, 5.5-7.2) and *Salmo labrax* x *Oncorhynchus mykiss* have increased growth rates relative to the parental species by day 200 post hatch (Akhan et al., 2011). While *O. mykiss* x *Salvelinus* sp. and *S. trutta* x *S. salar* hybrids have increased disease resistance (Maynard et al., 2016). Despite the potential advantages of the salmonid hybrids, their exploitation for aquaculture is uncommon due to high mortality during embryonic stages and up until external feeding (Bartley et al., 2000). Nonetheless, there are some reports that triploidization can increase the viability of salmonid hybrids (Scheerer & Thorgaard, 1983; Blanc et al., 2000; Blanc & Maunas, 2005). One potential reason for high mortalities during the larval stage may be abnormalities in digestive tract function and for this reason characterization of gastrointestinal tract development and the nutritional physiology of fish larvae can contribute to overcome such problems (Rønnestad et al., 2013).

Caspian brown trout, CBT, *Salmo trutta caspius* is one of the native cold-water species with high potential for aquaculture in the Caspian basin countries (Najafpour et al., 2019). The fish belongs to *Salmo trutta* and has a greater size, weight and growth

67 rate than other subspecies of the brown trout (Sedgwick, 1995). Although rainbow trout,
68 RT (*Oncorhynchus mykiss*) is one of the most important cultivated species worldwide
69 and its production has grown exponentially, it suffers from diseases outbreak (Olsen et
70 al., 2015). In contrast CBT is more resistant to disease but has a lower growth rate than
71 the RT and a hybrid of these two species may be a means of taking advantage of their
72 traits of interest for production. To the best of our knowledge there are no studies on
73 digestive tract ontogeny in salmonid hybrids. Therefore, in the present study, histology
74 and enzymatic assays (amylase, lipase and trypsin as indicators of carbohydrate, lipid
75 and protein digestion, respectively) were used to evaluate and compare the ontogeny of
76 the digestive tract in developing triploid hybrids (female RT and male CBT) and their
77 parents. Evaluating the morphology of gastrointestinal tract (GI-tract) (Gisbert, et al.,
78 2004) and digestive enzyme secretion (Kolkovski, 2001; Furne et al., 2005) can give
79 insight into when a fully functional GI-tract has developed and allow the adjustment of
80 feeding practices so they are appropriate for the developmental stage of the larvae.
81 Morever, characterizing the presence and levels of activity of the main digestive
82 enzymes during larval development provides valuable information for designing diets
83 with an appropriate composition (Mente et al., 2017).

84 **2. MATERIAL AND METHODS**

85 2.1 Experimental groups and breeding condition

86 Overall, four experimental groups were produced: I, diploid RT, *Oncorhynchus*
87 *mykiss*; II, diploid CBT, *Salmo trutta caspius*; III, triploid hybrids between female RT
88 and male CBT (*O. mykiss* ♀ x *S. t. caspius* ♂), and IV diploid hybrids between female

RT and male CBT (*O. mykiss* ♀ x *S. t. caspius* ♂). However, group IV of diploid hybrids (*O. mykiss* ♀ x *S. t. caspius* ♂) was excluded from further experimentation, due to high mortality during the embryonic and larval development.

Male and female brooders (10 of each from each species) were handled according to standard hatchery protocols to obtain the hybrids (Sedgwick, 1995). Average total length and weight of brooders were: RT: male 40.5±4.4 cm and 1207.5 ± 103 g, female 65.2 ± 5.7 cm and 3000.2 ± 150 g and CBT: male 40.2 ± 3.8 cm and 2100 ± 145 g, female 62.7 ± 3.3 cm 2818.6 ± 86 g. Ova and milt were collected by gently squeezing the abdomen of the fish anesthetized with clove powder (approx. 120-140 mg/l). *In vitro* fertilization was carried out using the dry method and adding 3–4 volumes of activating solution (125 mM NaCl, 30 mM Glycine, 20 mM Tris-HCl, pH=9) to the mix of milt and ova (Billard, 1983). Each population of eggs and milt for the respective crosses were generated from a pool of 500 grams of eggs and by adding sperm in a ratio of 1/100 (sperm volume/egg volume). The eggs were kept in plastic containers and underwent the water hardening process within 60 minutes post fertilization. Eggs were transferred to horizontal fiberglass incubator troughs (approx. volume = 200 liter; dimension (L×W×H) = 233×60×18 cm; tray capacity = 4 units; color = grey) and three replicate troughs were assigned to each experimental group (I, II, III, IV) using about 160 grams of fertilized eggs for each replicate (Fig. 1).

To incubate and culture fish eggs and larvae, a circulation system supplied with fresh water from a well, was used at the Isfahan university of Technology, Isfahan, Iran. Water (8 L/min) was supplied to the replicate treatment tanks by 3 header tanks of 2 m^3

111 and entered the troughs by gravity. During incubation, at 10 ± 1 °C, the egg trays were
112 covered with black plastic to prevent the effects of light, and any stress from
113 manipulation and displacement, was prevented. The water quality in the fish tanks was
114 monitored on a daily basis and maintained within the following limits: temperature, 9-
115 10 °C; dissolved oxygen, 8.9-9.5 mg/L; pH, 7.8–8.2; electrical conductivity, 482.2–
116 484.4 μs/cm; total hardness, 179–182 mg/L $CaCO_3$; total phosphate, 0.07–0.09 mg/L;
117 and dissolved organic carbon, 0.5–0.6 mg/L.

118 After incubation and hatching (RT and hybrid = 300 degree-day, CBT = 370
119 degree-day), the egg support trays were removed from the three replicate troughs and
120 the larvae were maintained under the conditions indicated above (Fig. 1). After 2/3 of
121 the yolk sac was absorbed, the larvae were fed extruded pellets (Biomar, crude protein:
122 60%; crude lipid: 10%; carbohydrates (NFE): 11.2%; Crude cellulose: 0.3%; ash:
123 12.2%; total phosphorus (P): 2.0%; gross energy: 20.4MJ/kg; digestible energy:
124 18.3MJ/kg; typical content of nitrogen (N): 9.6%, pellet size for 0.1-0.2g larvae = 0.4
125 mm; pellet size for 0.2-0.4g larvae = 0.6 mm). Larvae were fed seven times per day
126 before yolk sac absorption and twice a day after yolk sac absorption until 45 dph. Fish
127 were fed by hand *ad libitum* in each feeding.

128 2.2 Triploidy induction

129 Induction of triploids was carried following established procedures for RT and
130 CBT on a single batch of fertilized eggs, 10 min post-fertilization, by holding the eggs
131 at 30 ºC for 10 min (Arai & Wilkins, 1987; Dorafshan et al. 2008; Kalbassi et al., 2009).
132 The effectiveness of thermal shock in our study was evaluated at more than 95%. The

ploidy level was characterized by flow cytometry. Briefly, blood samples were taken from at least 10 fish (average weight = 0.6 g) in 75-mm heparinized capillary tubes. The blood cells were stained with the fluorescent dye, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma: 28718-90-3) that were diluted 1:10000 in a solution containing 0.146 M NaCl, 0.1 M Tris (pH 7.4), 0.2% bovine serum albumin, 10 pg/ml (DAPI), 0.021 M MgCl and 0.6% NonidetP-40 (Thorgaard et al., 1982). Then, the stained nuclei of the red blood cells were detected using a FL2-H fluorescent detector and the fluorescent density, determined using the horizontal axis of the flow cytometer histogram, to establish the relative DNA content per cell nucleus.

2.3 Sample collection

Larvae were collected from each replicate trough during the experiment at: 3, 6, 9, 12, 15, 18, 21, 26, 31, 35, 40 and 45-days post hatch (dph). Sampling occurred before feeding at approximately the same time each day (17.00 h) in each of the experimental groups. A sample of 10 larvae was collected for GI-tract histology and another sample of 10 larvae was collected for enzyme activity from each of the replicate troughs (i.e. 6 pools of 10 larvae/experimental group corresponding to 60 larvae) were collected per timepoint. Larvae for histology were anesthetized in ice-cold water, fixed in 10% neutral buffered formalin (pH=7.4) and stored at 4 ºC until analysed (3-6 were analyzed at each sampling time) (Fig. 1). For growth analysis, 10 larvae per experimental group were anesthetized and weighed (g) at each sampling times. Larvae for the enzyme assays, were anesthetized in ice-cold water, excess moisture was removed and then they were snap-frozen in liquid nitrogen and stored at -80 ºC. The enzyme assays were

155 performed at CCMAR, Portugal and so before sample shipping they were lyophilized
156 at -60 ºC at Isfahan University of Technology, Iran to ensure sample stability.
157 2.4 Growth performance and survival rate
158 Survival rates were evaluated at the eyed egg, hatching, and active swimming
159 (swim bladder inflation) stages. The specific growth rate (SGR) was calculated
160 according to the following formula:
161 SGR % = 100 × (Ln (average terminal body weight)-Ln (average initial body weight))
162 / number of test days.
163 2.5 Histological analysis
164 Larvae between 3 and 18 dph were processed directly for histology. Larvae
165 between 21 and 45 dph were decalcified before embedding in paraffin wax. Larvae
166 were decalcified by washing them in sterile water 3 x 10 minutes followed by
167 immersion in a solution of 0.5 M EDTA, pH 8.0. The decalcifying larvae were kept in
168 the dark at room temperature with constant, gentle agitation and the EDTA solution was
169 changed every 2 days over 5-7 days. After EDTA decalcification, samples were washed
170 in sterile water for 3 x 30 minutes and stored in 70% ethanol. For paraffin embedding
171 all larvae (3 – 45 dph) were dehydrated in a graded series of ethanol (70, 90 and 100%),
172 saturated in xylene and impregnated with low melting point paraffin wax (58ºC,
173 Histosec, Merck). Serial sagittal sections (5 µm thick) of wax embedded larvae were
174 prepared using a manual rotary microtome (Leica RM 2135, Germany). Sections were
175 mounted on 3-aminopropyltriethoxysilane (APES) coated glass slides, dried overnight
176 at 37 °C and then stored until staining. At least 3 slides from 3 individuals of the same

177 stage were analyzed after staining with haematoxylin and eosin (H&E). The
178 morphological characteristics of the gastrointestinal tract was analyzed using a
179 microscope (Leica DM2000) coupled to a digital camera (Leica DFC480) linked to a
180 computer for digital image analysis.

181 2.6 Enzyme assays

182 Preliminary studies were carried out to optimize the extraction of protein from
183 lyophilized larvae. The buffers tested for protein extraction were RIPA (150mM NaCl,
184 50mM Tris HCl, 1% Triton x100, pH 8), phosphate buffer (50 mM, pH 8.4) and Tris
185 buffer (50 mM, pH 8.4). The highest protein yield was obtained with Tris buffer (50
186 mM, pH 8.4), which was adopted for all extracts. To standardize the extraction protocol,
187 from a pool of 10 lyophilized larvae, 25 mg was taken for each crude extraction sample
188 and solubilized in 500 µl ice-cold Tris buffer (50 mM, pH 8.4), vortexed and
189 homogenized for 10 s (UltraTurrex, Spain), and maintained on ice for 10 minutes.
190 Samples were centrifuged (10,000 rpm) at 4 °C for 10 minutes and the supernatant (300
191 µl of larval extract) collected and stored at -80 °C until analysis.

192 *Total protein* was quantified in triplicate using a Bradford assay (Bio-Rad, USA)
193 scaled down for a microplate. Briefly, 5 µl of the supernatant from the crude extract and
194 250 µl of the dye reagent 1x were added to each microplate well. After 5 minutes of
195 incubation in the dark, the absorption was read at 595 nm using a UV/Vis
196 spectrophotometer (Thermo Fisher Scientific Oy, Ratastie 2, Fl-01620, Vantaa, Finland).
197 The total protein of the crude extract was expressed as mg/g dry weight of larvae.
198 Bovine Serum Albumin was used to prepare a standard curve (Quick Start BSA

199 Standard Set #5000207, Bio-Rad, USA) and the concentration of protein in samples
200 was calculated and expressed as mg/g dry weight larvae.

201 *Alpha-amylase activity* was analyzed using the 3,5-dinitrosalicylic acid (DNS)
202 method (Bernfeld, 1951; Worthington, 1991). A starch substrate (1% w/v) (Sigma-
203 Aldrich-33615) solution was prepared by mixing it in phosphate buffer (20 mM
204 Na2HPO4, pH 6.9) and boiling for 15 minutes with constant mixing. The assay was
205 optimized for a 96-well microplate, and each reaction contained 55 µl of the 1 % starch
206 solution and 25 µl of the larval extract and was incubated at 37 °C for 10 min. Colour
207 development was carried out by adding 50 µl of 1 % dinitrosalicylic acid (DNS) to each
208 reaction and boiling for 5 mins at 100 °C. The reaction mixture was then cooled on ice
209 for 5 minutes and 155 µl of distilled water was added to each well. Blank reactions were
210 prepared in the same way but without adding the extracts containing the enzyme. The
211 absorbance was read at 540 nm using a UV/Vis spectrophotometer (Thermo Fisher
212 Scientific Oy, Ratastie 2, Fl-01620, Vantaa, Finland). A standard curve of monohydrate
213 maltose (0.9-4.5 µM/ml) was prepared using the same procedure as for the samples but
214 without adding starch. The specific activity of α-amylase is represented as µmol
215 maltose /min /mg protein.

216 *Lipase activity* was determined using p-Nitrophenyl laurate as the substrate
217 (Winkler & Stuckmann, 1979). One unit of lipase produces 1 µmol of p-nitrophenyl
218 /min under the specified reaction condition. The assay was optimized for a 96-well
219 microplate. Each reaction contained 25 µl of the substrate (420 µM p-Nitrophenyl
220 containing SDS and Triton-X 1 %) and 25 µl of 0.1 M Tris-HCl buffer (pH 8.2) and 10

µl of larval extract and was incubated at 35 °C for 30 min. The change in absorbance of the reaction was continuously monitored over 10 minutes at 410 nm. A standard curve was generated using p-nitrophenol (range 5-60 µM) and following the same procedure as for the samples. 0.1 M Tris-HCl buffer (pH 8.2) was used as the blank. Lipase activity (U/ml) was determined using the following formula:

Lipase activity (U/ml) = $\Delta A \times Vf / \Delta t \times Vs \times e$

Where, ΔA is the difference in absorbance between the beginning and end of the assay, Vf is the final volume (ml), Δt is the duration of the assay, Vs is the volume of the sample (ml) and e is the slope of the standard curve. Lipase activity is expressed as mU/ mg protein.

Trypsin equivalent activity was determined using a modification of the method developed by (García-Carreño, 1992) for evaluation of total protease activity. Reactions contained 100 µl of larval extract and 100 µl azocasein (0.5% in 100 mM Tris buffer, pH 8). Reactions were incubated for 10 min at 25 °C and were stopped by adding 100 µl of 10 % TCA and incubating at 25 °C for a further 15 min. The supernatant was collected by centrifugation (1200 relative centrifugal force for 5 min at 4 °C) and 100 µl of the supernatant was added to 100 µl of 1 N NaOH in a 96-well microplate. Absorbance was recorded at 450 nm. For the standard curve, trypsin (Sigma T-7409, 1000 – 2000 BAEE per mg solid) was diluted in physiological buffer (0.1 M Tris-HCl, 0.02 M CaCl2, pH 7.9) and was subject to the same reaction conditions as outlined above but substituting the larval extracts for trypsin (range: 0.54-7 mg/ml). Trypsin equivalent activity is expressed as U/mg protein of larvae. One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolyzes azocasein resulting in an increase in 0.001 units of absorbance/minute/ ml (Candiotto et al., 2018; Campos et al., 2019).

2.7 Statistical analysis

To analyze growth and the activity of the digestive enzymes in each experimental group with time/age and also to compare different groups (RT, CBT and the triploid hybrid) at each age/sampling time two-way analysis of variance was applied using SigmaPlot (version 14; Systat Software GmbH, Erkrath, Germany) followed by a Holm-Sydak post-hoc test. Statistical significance was taken as $p < 0.05$. The results are reported as the means ± standard error of the mean, *SEM*.

3. RESULTS

3.1 Survival and growth performance

The survival rate at the eyed egg, hatching and active swimming or first feeding stages, was highest for RT and lowest for the triploid hybrid (Table 1). The growth performance of the larvae was evaluated across the 45 days of the study and the final average weight (g) and SGR was highest for RT and triploid hybrids and lowest for CBT ($p < 0.05$; Table 1, Fig. 2).

3.2 Histology

The main morphoanatomical changes of the GI-tract of RT, CBT and their hybrid were identified by histology up to 45 dph (Table 2). Yolk sac absorption was considered as a putative marker for the complete transition from endogenous to exogenous energy reserves. Apart from the timing of the development of the digestive organs, the general structure and the main ontogenetic steps of GI-tract development in the studied groups were similar and showed increasing complexity as the larvae grew (Fig. 3A, B, C). The yolk sac and vitelline syncytium were observed from 3 to 18 dph in RT, while in the triploid hybrid and CBT, they were visible until 31 dph (Fig. 3A, B, C). In all studied

groups, the GI-tract was a relatively simple tubular structure at hatching (Fig. 3A). A rudimentary esophagus was distinguishable from other regions at 3 dph and was well-developed with a large diameter and many mucosal folds containing abundant goblet cells at older ages (e.g. 40 dph, Fig. 3C).

At 3 dph, the stomach was distinguished as a sack-like swelling of the intestine and at the anterior and posterior regions, the forming valves created a constriction where it joined the esophagus and proximal intestine, respectively (Fig. 3A). The developing cardiac sphincter was evident at the junction of the esophagus and stomach and the pyloric sphincter at the junction of the stomach and anterior intestine. On day 6, the stomach appeared as a small swelling of the intestine but as it developed two main regions (cardiac and fundus) were evident and it gradually curved and assumed a characteristic U-shaped. The stage at which stomach flexion occurred was 15 dph in RT and 18 dph in the triploid hybrid and CBT (Fig. 3A, B). The triploid hybrid and CBT reached a similar developmental stage at 31 dph indicating they have a similar developmental rate (Fig. 3B, C). However, from 31 dph to 45 dph the formation of a well-developed U-shaped stomach and pyloric caeca occurred more rapidly in the triploid hybrid than in CBT (Fig. 3C). Pyloric caeca were evident at 26 dph in RT, at 35 dph in the triploid hybrid and at 40 dph in CBT (Fig. 3B, C). The development of the pyloric caeca was the last detected morphoanatomical change in the GI-tract.

At 3 dph, the folding of the absorptive surface of the intestine was not evident although signs of primitive folds were observed (Fig. 3A). As the larvae grew, the gut lengthened, and the intestinal lumen was occupied by undulating mucosal folds that

291 became more obvious at 6 dph. From 15 dph, abundant mucosal folds were evident in
292 all groups indicating the digestive and absorptive surface was increased. In general, the
293 morphoanatomy of the lumen of the GI-tract was similar between RT, triploid hybrid
294 and CBT until 15 dph (Fig. 3A). After 15 dph, the rate of differentiation of the
295 esophagus and intestine differed between the species, but the most notable difference
296 was found in the timing of stomach formation and the development of the pyloric caeca.
297 Overall, the developmental rate of the GI-tract in the hybrid and RT was more similar
298 compared to CBT which had a slower developmental rate. Furthermore, the GI-tract
299 development rate in larvae paralleled the whole-body growth rate for RT, CBT and the
300 hybrid.

301 **3.3 Biochemical and enzyme assay**

302 Total protein

303 The relative concentration of protein (mg/g dry weight of larvae) increased with
304 increasing age in all experimental groups and reached a maximum (p<0.05) at 15 dph
305 for RT (Fig. 4), 35 dph for the triploid hybrid (Fig. 4) and at 21 dph for CBT (Fig. 4).
306 Once the relative protein content of larvae reached a maximum, no further rise in the
307 protein content (mg/g dry weight of larvae) was observed in the RT, CBT or triploid
308 hybrid (Fig. 4)

309 Trypsin

310 Between 3 and 26 dph, trypsin activity (measured in trypsin equivalents) was low
311 in the developing larvae of all studied groups. Trypsin activity then increased
312 exponentially with a significant peak (p<0.05) at 35 dph for RT, at 45 dph for the triploid

313 hybrid and at 31 dph for CBT (Fig. 4). After the peak in trypsin activity a significant
314 decrease (p<0.05) occurred at 35 and 40 dph in CBT and RT respectively (Fig. 4).
315 Amylase
316 Higher levels of amylase activity were detected at 40 dph for RT in comparison
317 to the other groups (p<0.05; Fig. 4). In the triploid hybrid, amylase activity was stable
318 across the 45 days of the experiment and the average level of activity, 3.78 mU/mg
319 protein, was mid-point between the levels of activity measured for RT (4.86 mU/mg
320 protein) and CBT (3.58 mU/mg protein) (Fig. 4). The highest amylase activity occurred
321 at 31 dph in the CBT (p<0.05; Fig. 4). In all studied groups, a sharp decline in the
322 enzyme activity were measured immediately after the highest point (Fig. 4).
323 Lipase
324 A continuous decrease in lipase activity levels were observed in RT from hatching
325 until 35 dph (Fig. 4). The decreasing trend in lipase activity was also detected in the
326 triploid hybrid where a minimum was recorded at 45 dph (Fig. 4). In CBT, the highest
327 measurable level was observed at 18 dph and then it decreased to a minimum at 45 dph
328 (Fig. 4).
329 **4. DISCUSSION**
330 The survival rate and digestive tract ontogeny between RT, CBT and their triploid
331 hybrids were analyzed. The mortality rate of triploid hybrid (RT ♀ x CBT ♂) was higher
332 than the parental species during the incubation phase before and during hatching and
333 this has previously been reported in salmonid hybrids (Blanc et al., 2000; Blanc &
334 Maunas, 2005). Fujiwara et al. (1997) suggested that chromosome imbalance and/or

335 elimination could cause the lack of viability in some salmonid interspecific hybrids and
336 could be improved by triploidy induction.

337 The histology results suggest obvious folding of the pharynx and esophagus and
338 the presence of abundant goblet cells as indicators of a well-developed esophagus at
339 older ages (e.g. 40 dph). Increasing longitudinal folds and goblet cells along the length
340 of the esophagus with age have been observed in other studies (Yang et al., 2010) and
341 have previously been linked with functional changes in the anterior part of the digestive
342 tract (Hamlin et al., 2000). The early formation of a simple buccopharynx and
343 esophagus a few days post hatch in all the larval fish studied (CBT, triploid hybrid and
344 RT) corroborates the results of previous studies on RT (Sarieyyüpoğlu et al., 2000) and
345 in other salmonid species e.g. Atlantic Salmon *Salmo salar* (Sahlmann et al., 2015).
346 Observations from the present and previous studies suggest that during early larval
347 development the increase in diameter of the esophagus and its degree of folding can be
348 used as a morphological marker of GI-tract development in the post-feeding stages.

349 The developing stomach in triploid hybrids and their parental species was evident
350 soon after hatching suggesting they are not altricial-gastric fish (Rønnestad et al., 2013;
351 Gomes et al., 2014) since differentiation of the stomach occurred before the onset of
352 first feeding. The histological observations of the GI-tract suggest that the stomach was
353 most likely functional in all fish groups before the yolk sac was totally resorbed and
354 exogenous feeding was initiated, even though it was not fully developed. This is in line
355 with previous observations in *S. salar*, *Oncorhynchus masou* and *Oncorhynchus keta*
356 (Sahlmann et al., 2015). The early appearance of a stomach in RT corroborates

observations from other studies (Sarieyyüpoğlu et al., 2000) but this is the first time that the ontogeny of the stomach has been reported and shown to be conserved in the CBT and the hybrid. Adult salmonids have a siphonal or U-shaped stomach that is common among the Osteichthyes and is linked to their feeding strategy (Wilson & Castro, 2010).

The results of the present study revealed that the time at which the GI-tract folded to the more efficient and functional U-shaped stomach differed between the studied groups. The differences in the timing of stomach formation may explain the significant differences in the growth rate of the larvae between the hybrids and non-hybrids. Moreover, the pyloric caeca, another indicator of digestive tract function, appeared at different time points in the older larvae of the hybrid and non-hybrid fish studied (Table 2). The relevance of the pyloric caeca is their importance as possible structures for digestion and nutrient uptake (sugar, amino acid, and dipeptide) and their appearance gives an indication of digestive tract functionality as has been shown in previous studies (Blier et al., 2007). The obvious development of these structures in terms of morphology was well matched with a well-developed U-shaped stomach. This suggests the pyloric caeca may have an important role in the secretion of digestive enzymes such as trypsin while when the gastric glands become fully functional and secrete hydrochloric acid and pepsin into the stomach the role of trypsin in the stomach is limited. This idea is supported by studies of Atlantic cod in, which it was shown that the combined effect of trypsin activity and the size of pyloric caeca resulted in increased digestive capacity (Blier et al., 2007).

High mortality can occur at the transition to exogenous feeding and this is a critical stage in larval development and the timing of the changes in morphology of the mouth and digestive tract is decisive (Makrakis et al., 2005). The results of the present comparative study suggest that adaptation to dry food before complete resorption of the yolk sac may be a key factor in decreasing mortality at weaning as in RT with the fastest GI-tract development, the lowest mortality was observed. Although, the results could suggest that higher mortality of the triploid hybrid than CBT at the swimming stage was the results of hybridization rather than an underdeveloped digestive tract.

A short steady-state phase after hatching and then an exponential increase in trypsin activity linked to start-feeding seems a common event during fish larval development (Lemieux et al., 2003; Sahlmann et al., 2015; Teles et al., 2019). In general, from hatching to metamorphosis approximately four-phases have been proposed for trypsin activity development in fish larvae (reviewed by Rønnestad et al., 2013). In phase I, trypsin increases in the yolk-sac stage to the start of exogenous feeding; in phase II, trypsin activity decreases and this is a critical stage, which can lead to poor growth and high mortality rates; in phase III, trypsin production is in balance with the food supply and in the last phase (IV) trypsin activity drops at the beginning of metamorphosis. The results obtained in the present study did not comply with the proposed model for trypsin activity development in marine fish larvae probably due to their ontogenetic diferences (Rønnestad et al., 2013). The results suggest that phase II did not occur and that the highest level of trypsin activity and subsequent decrease (phase IV) may be associated with the first metamorphic transition to parr (after

absorption of the yolk sac) (Parkyn & Hawryshyn, 2000). The decrease in trypsin is proposed to be linked to the formation of the gastric glands and onset of pepsin activity (Chen et al., 2006; Ma et al., 2014; Teles et al., 2019).

Comparison of the histological sections and the trypsin enzyme activity obtained in the present study revealed that the morphoanatomical appearance indicative of a fully functional stomach coincided with the time that trypsin reached its lowest value in all the studied groups. In the Atlantic salmon it was also observed that the formation of a fully functional stomach was associated with the decline in trypsin activity, which reached a minimum at 80 dph when pepsinogen transcripts were high (Sahlmann et al., 2015). Based on the results of the Atlantic salmon and the results from the present study we propose that the decrease in trypsin activity (which has been less considered in ontogeny studies) is linked with the formation of a fully functional stomach and possible ability to digest high-protein formulated diets. The peak in trypsin may signal the full development of the exocrine glands/secretory process of the trypsinogen producing pancreas. This is further supported by observations that pancreas zymogen granule abundance was correlated with trypsin activity in homogenized whole larvae of seabass (*Dicentrarchus labrax* L) (Beccaria et al., 1991) and that the gradual increase in trypsin expression and activity in the developing bullseye puffer fish *Sphoeroides annulatus* coincided with the development of the pancreas (Garcia-Gasca et al., 2006).

The gradual rise in the specific activity of amylase during development coincided with weaning to dry food in RT and CBT and corroborates observations in other fish larvae that the development of the digestive system is stimulated by food (Zouiten et

al., 2008). The reduction in amylase activity in RT and CBT with age may be linked to carnivorous feeding as previously reported for other carnivorous fish species such as seabream and other marine species (Khoa et al., 2019; Yúfera et al., 2018). No progressive increase in amylase was observed in the triploid hybrids during early development, suggesting a difference in the carbohydrate metabolism pattern in this particular group compared to RT and CBT. It seems amylase activity of the triploid hybrid is more influenced by the maternal genome (RT) as the average activity of amylase was higher than in CBT and this may explain the similar growth rate between the triploid hybrid and RT. The peak in amylase, another pancreatic enzyme, is similar to what was observed for trypsin in the RT and CBT and further substantiates the idea that the pancreas is fully functional.

In contrast to amylase, lipase activity was similar in all experimental groups. The high levels of lipase early in ontogeny was most likely extra-intestinal and this probably reflects the dependency of larvae on lipids mobilized from the yolk. A similar pattern of lipase activity was observed in the Asian sea bass (Srichanun et al., 2013), longfin yellowtail (Teles et al., 2019) and Persian sturgeon (Gilannejad et al., 2019) where the lipase activity was increased in the first few days post hatch and then decreased as age increased. The switch from dependency on the yolk to an external food source presumably explains the progressive reduction in lipase to a minimum at metamorphosis in RT, CBT and the hybrid.

CONCLUSION

This study highlighted some histological indicators of a functional GI-tract in

445 salmonids such as the increase in folding and goblet cells in the pharynx and esophagus,
446 U-shaped stomach and pyloric caeca. The GI-tract development and digestive enzyme
447 (trypsin, lipase and amylase) activity in triploid hybrids was more similar to RT than
448 CBT. This may be explained by the greater genetic contribution of the female since the
449 offspring acquire an extra maternal chromosome set through retention of the polar body.
450 In general, RT and CBT showed the highest and lowest growth rates, respectively. The
451 data suggests that trypsin and amylase activities during ontogeny were induced by
452 exogenous feeding and that the subsequent decrease in their activity may be linked to
453 the complete transition to exogenous feeding and metamorphosis. There was also
454 indication of species/hybrid specific enzymatic patterns and activity levels such as the
455 amylase pattern in triploids. The differing patterns of morphoanatomical development
456 and digestive enzyme activities in the GI-tract of the hybrid, RT and CBT may be useful
457 to establish the most appropriate timing for exogenous feeding but also to optimize the
458 composition of dry feeds.

459 Production relevant insights into the effect of triploidization on trait inheritance
460 such as growth and digestive capacity in triploid hybrids of RT and CBT is reported for
461 the first time. The results indicate that the future use of triploid hybrids in aquaculture
462 is promising. However, the low survival of hybrids at the early stages is an explicit
463 limitation. Therefore, more efforts will be required before the hybrids of these species
464 can be fully exploited for aquaculture.

465 **ACKNOWLEDGEMENT**

466 The authors acknowledge comparative endocrinology and integrative biology group

members of CCMAR (Centro de Ciências do Mar, Universidade do Algarve Portugal) for their kind assistance. Work at CCMAR was funded by the Portuguese Foundation for Science and Technology (FCT) project UID/Multi/04326/2019. The Department of Natural Resources of Isfahan University of Technology provided fish breeding and culture facilities and support from Iran's Ministry of Science, Research and Technology.

CONFILICT OF INTEREST: The authors declare that they have no conflict of interest.

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DATA AVAILABILITY STATEMENT

Data available on request from the authors: We declare that the data which support the findings of this study are available from the corresponding author upon reasonable request.

TABLE 1. Survival and growth rates of the rainbow trout, *Oncorhynchus mykiss,* triploid hybrid and Caspian brown trout, *Salmo trutta caspius* between hatching and 45-days post hatching

Groups	Rainbow trout	Triploid hybrid	Caspian brown trout
Eyed egg survival (%)	93.21±1.05^{a}	33.33±1.18^{b}	90.12±1.08^{a}
Hatching survival (%)	96.13±1.01^{a}	83.14±1.09^{b}	95.26±1.04^{a}
Active swimming (%)	85.12±0.82^{a}	55.18±1.21^{b}	82.28±1.12^{a}
Initial average weight (g)	0.089±0.03^{b}	0.084±0.05^{bc}	0.103±0.01^{a}
Final average weight (g)	0.29±0.30^{a}	0.20±0.01^{a}	0.17±0.09^{b}
Specific growth rate (SGR) (%)	2.76±0.32^{a}	2.08±0.21^{a}	0.53±0.03^{b}

* Triploid hybrids: female rainbow trout x male Caspian brown trout

In each row, groups with different superscript are statistically different ($p < .05$).

Data are presented as means ± *SEM*.

631 **TABLE 2.** Timing of the gastrointestinal tract morphoanatomical changes in
632 salmonids (present study compared to some previously published works)

GI-tract Characteristic	Rainbow trout†	Triploid hybrid†	C. brown trout†	Rainbow trout‡	Atlantic salmon‡*
Mouth opening	3 dph	3 dph	3 dph	3 dph	7 dph
Esophagus differentiation	3 dph, obvious folding at later stage of hatching	3 dph, obvious folding at later stage of hatching	3 dph, obvious folding at later stage of hatching	3 dph, mucosal folds at 13 dph and fully formed at 17 dph	7 dph
Intestine folding	Gentle fold at 3 dph; developing at 6 dph; well-formed 15 dph	Gentle fold at 3 dph; developing at 6 dph; well-formed 15 dph	Gentle fold at 3 dph; developing at 6 dph; well-formed 15 dph	-	Forming at 7 dph; simple, folded mucosa along the entire intestine at 27 dph
Pyloric caeca expansion	Obvious expansion of intestine from 26 dph, yolk sac absorbed	Obvious expansion of intestine from 35 dph, yolk sac absorbed	Obvious expansion of intestine from 40 dph, yolk sac absorbed	-	Pyloric caeca buds at 27 dph; more developed at 46 dph (one week before yolk sac absorption)
U-shaped stomach	Begins to form at 15 dph; well-shaped at 26 dph	Begins to form at 18 dph; well-shaped at 35 dph	Begins to form at 18 dph; well-shaped at 40 dph	-	Well-shaped at 46 dph,

633 † Rainbow trout, *Oncorhynchus mykiss*, triploid hybrid (*O. mykiss* ♀ x *S. t. caspius* ♂), and Caspian
634 brown trout, *Salmo trutta caspius* in the present study. ‡ Rainbow trout (Sarieyyüpoglu et al., 2000) and
635 Atlantic salmon, *Salmo salar* (Sahlmann et al., 2015). * First sample taken at 7 dph, eggs and alevins at
636 7–8°C, from start-feeding at 11–12°C.

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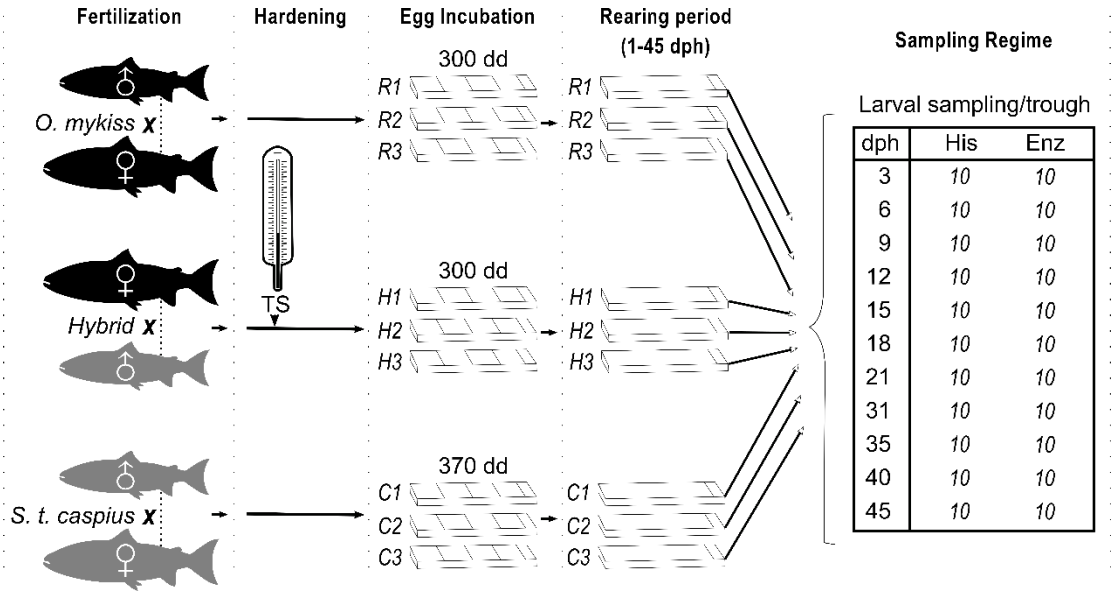
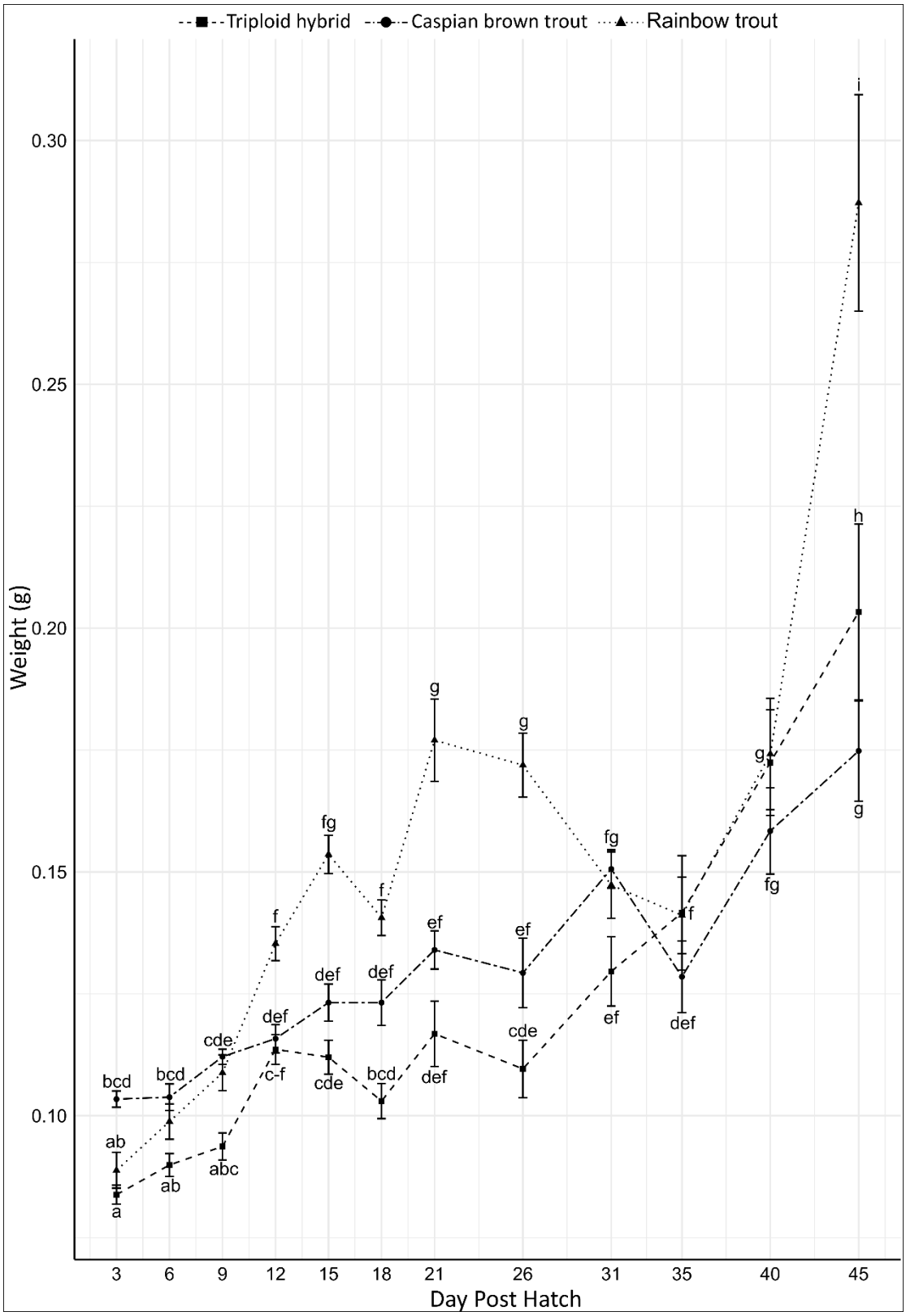


Figure 1. A schematic of the experimental design. The crosses (*X*) performed between *Oncorhynchus mykiss (O. mykiss) and Salmo trutta caspius* (*S. t. caspius*) to generate the three main experimental groups is indicated. The triploid hybrid was produced by thermal shock (TS) at 30 ºC for 10 min and was applied 10 min post-fertilization. The egg incubation period is expressed in degree days (dd) for each group. Triplicate horizontal troughs represent the replicates of each experimental group: Rainbow trout (R1, R2, R3); Triploid hybrid (H1, H2, H3); Caspian brown trout (C1, C2, C3). The number of larvae sampled for histology (His) and enzymes assays (Enz) per replicate /trough are indicated in the table for each sampling timepoint 3, 6, 9, 12, 15, 18, 21, 26, 31, 35, 40 and 45 dph (days post hatch).



650

651 **Figure 2.** Weight of rainbow trout, triploid hybrid (rainbow trout ♀ x Caspian brown
652 trout ♂) and Caspian brown trout (mean ± SEM) from 3 to 45 days post hatch at 10 ±
653 1 °C. Different letters indicate significant difference (p<0.05, two-way ANOVA, Holm-
654 Sidak a posteriori test, n = 8 -10 per group).

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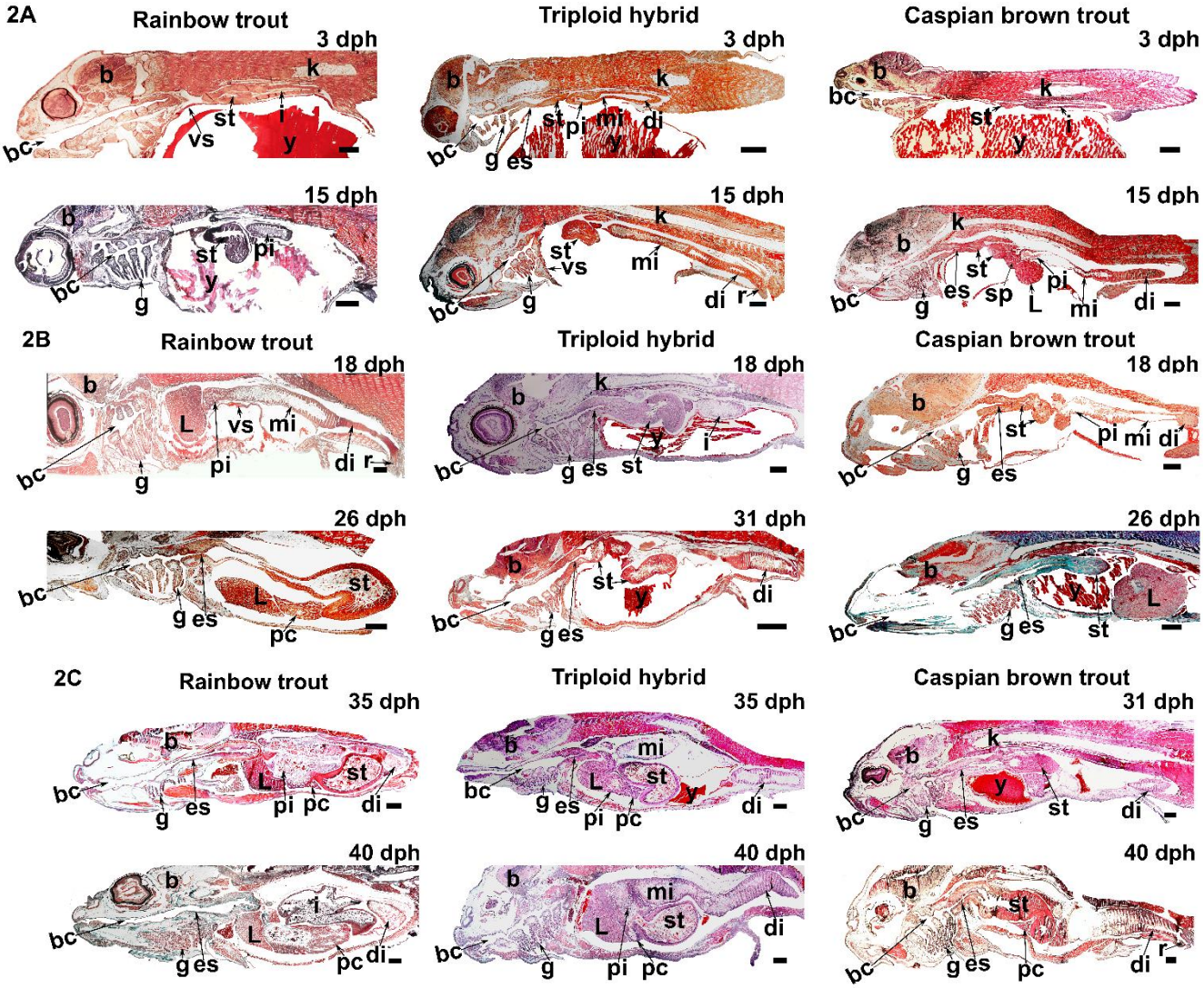
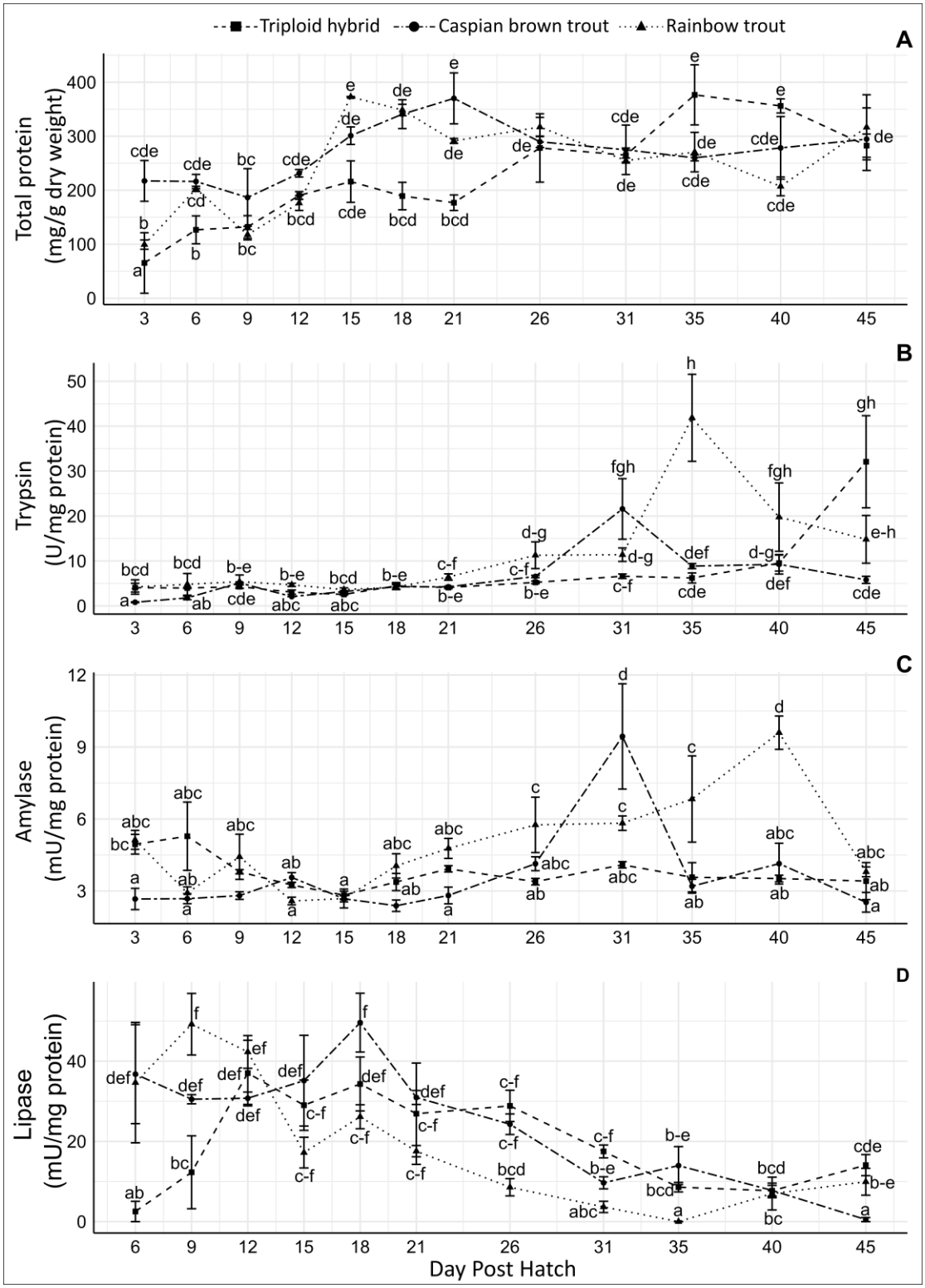


Figure 3. Sagittal sections of rainbow trout, triploid hybrid (rainbow trout ♀ x Caspian brown trout ♂) and Caspian brown trout at 3-15 post hatch (dph) (3A), 18-31 dph (3B) and 35-40 dph (3C) stained with haemotoxylin and eosin. The developing gastrointestinal tract is clearly visible (3-6 larvae per time point were analyzed). Abbreviations: bc, buccopharyngeal cavity; es, esophagus; i, intestine; pi, anterior intestine; mi, mid-intestine; di, posterior intestine; L, liver; pc, pyloric caeca; st, stomach; vs, vitelline syncytium; y, yolk; k, kidney; g, gill; b, brain; h, heart; r, rectum. Scale bar: 300 μm.



673

674 **Figure 4**. **A** - Total protein (mg/g dry weight); **B** – Trypsin equivalent (U/mg protein);
675 **C** – Amylase (mU/mg protein); and **D** - Lipase (mU/mg protein) (mean ± SEM) of
676 rainbow trout, triploid hybrid (rainbow trout ♀ x Caspian brown trout), and Caspian
677 brown trout between 3 to 45 days post hatch. At each time point, 3 pools of 10 larvae
678 each were used in enzymes assays. For each group of fish, values with different letters
679 indicate significant difference (p<0.05, two-way ANOVA, Holm-Sidak a posteriori test,

n = 3).