The effect of algal turbidity on larval performance and the ontogeny of digestive enzymes in the grey mullet (*Mugil cephalus*)

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Abstract

A study comprised of two trials was carried out to determine and compare the effects of water turbidity produced by live microalgae and inert clay particles on the larval rearing of grey mullet (*Mugil cephalus*). ITrial 1 evaluated the effect water turbidity generated by microalgae on grey mullet larval performance and digestive enzyme activities along ontogeny. In particular, two microalgae (*Nannochloropsis oculata* and *Isochrysis. galbana*) were used, whereas water turbidity levels (0.76 and 1.20 NTU) and a non-microalgae control (0.26 NTU) were tested from 2 to 23 dph grey mullet larvae (5 treatments). The higher turbidity (1.2 NTU) larvae (5 dph) consumed markedly ($P < 0.05$) more rotifers than other treatment fish, independently of the microalgae type. Alkaline phosphatase activity was *ca.* 8 times higher and α-amylase activity increased 5.3 times in 79 dph fish compared to 40 dph individuals. The ratio of alkaline phosphatase and leucine-alanine aminopeptidase indicated gut maturation occurred around 61 dph, as well as a transition from carnivorous to omnivorous feeding habits. Trial 2 compared the most effective *N.occulata* produced turbidity level (1.2 NTU) with the identical water turbidity produced by inert clay on larval performance. *M. cephalus* larvae exposed to high algal turbidity demonstrated superior performance ($P < 0.05$), in terms of rotifer ingestion, dry weight gain and survival, in comparison to cohorts reared under the clay treatment as well as a lower microalgae produced turbidity. These findings suggested that water algal turbidity is not the dominant factor determining improved grey mullet larval performance.

1. Introduction

In the commercial rearing of marine fish larvae, tanks are frequently "greened" with microalgae such as *Nannochloropsis oculata* or *Isochrysis galbana*. It is widely believed and demonstrated that the provision of these algae into the tanks significantly improves larval performance and has become an inseparable part of commercial rearing protocols in fish farms around the Mediterranean basin

(Papandroulakis et al., 2002; van der Meeren et al., 2007; Bentzon-Tilia et al., 2016). On the other hand, it remains speculative how algal supplementation contributes to larval growth and survival or whether this benefit is species-specific. The biochemical composition of algal species (e.g. fatty acids) varies considerably and it is entirely possible that particular compounds secreted from the algal cell (e.g. polysaccharides) and/or are released during digestion might stimulate the immune system or enhance the digestive process in larvae (Hemaiswarya et al., 2011). In addition, water turbidity from specific algal concentrations may modify the light milieu for larvae, providing optimal backlighting for larvae to facilitate live prey identification (e.g. rotifers), foraging behavior and thereby enhancing hunting success (Rocha et al., 2008).

The grey mullet (*Mugil cephalus*) is an economically important euryhaline and eurythermal species contributing to sizable fisheries of estuarine and coastal regions in a variety of countries. It has been traditionally farmed extensively in ponds and enclosures in the Mediterranean region, South East Asia, Korea, Taiwan, China, Japan and Hawaii (FAO, 2018) and has been recently recognized as a highly valued candidate for more intensive aquaculture (Whitfield et al., 2012). Nevertheless, the rearing of the early developmental stages of grey mullet and the mass production of robust, fast growing juveniles remains an obstacle to the successful domestication of this species.

A study comprised of two trials was carried out to determine and compare the effects of water turbidity produced by live microalgae and inert clay particles on the larval rearing of grey mullet. More specifically, the aims of the first trial were to (1) investigate the effect of microalgae produced turbidity in the rearing tank on larval performance, in terms of prey capture efficiency, growth and survival, as well as digestive tract enzyme ontogeny. (2) Determine whether turbidity effect varies with microalgae type (*Nannochloropsis oculata* vs. *Isochrysis galbana*) and concentration (cells ml^{-1}). The aim of the second trial was to elucidate whether water turbidity or algal biochemical composition were the dominant factor promoting improved larval performance in this species.

2. Material and methods

2.1 Trial 1: Evaluation of the effect of water turbidity from two species of live microalgae on larval and juvenile grey mullet performance and ontogeny of the digestive tract functionality

Grey mullet eggs (gastrula stage) were stocked in fifteen 1.5 m^3 V-tanks (100 eggs l^{-1}) in a flow through system where filtered (10 µm), UV-treated, 40 ‰ seawater (25 ºC) entered from the tank bottom at a rate of two tank exchanges per day. Two experimental water turbidity levels A (0.76 NTU) and B (1.20 NTU) were tested using two microalgae species (*N. oculata* and *I. galbana*) and compared to non-microalgae supplementation C (control; 0.26 NTU) in 2 to 23 days post hatch (dph) grey mullet larvae. This meant that each of the 5 treatments; C (Control), Nanno A (*N. oculata*-turbidity A), Iso A (*I. galbana*-turbidity A), Nanno B (*N. oculata*-turbidity B), Iso B (*I. galbana*-turbidity B) were investigated with three replicate tanks per treatment. The different microalgae turbidity treatments and their concentrations are listed in Table 1.

The hatching rate (%) of stocked grey mullet eggs and survival of the pre-larva at the end of the day of hatching (0 dph) were calculated by placing a fertilized egg, at the gastrula stage, in each of 12 wells (5 mL) in each of three plastic well plates. The plates were covered and placed in a temperature controlled incubator until hatching where the emerging larvae and surviving newly hatched larvae at the end of 0 dph were counted. After hatching in the tanks, water salinity was progressively lowered to 25 ‰ and the flow rate reduced to one tank water exchange per day. Lighting over the tanks provided 500 lux (14 h light day^{-1}) at the water surface. At 2 dph, once the yolk sac was depleted, the eyes pigmented and the mouth and anus opened. From this point until 16 dph, grey mullet larvae were offered rotifers (*Brachionus rotundiformis*) that were previously enriched with taurine (600 mg L^{-1}) and essential fatty acids (Red Pepper™, Bernaqua, Belgium) for 12 and 8 h, respectively. After

feeding rotifers exclusively to 16 dph, larvae were co-fed with enriched rotifers and *Artemia* metanauplii (Red Pepper™) until 24 dph. From 25 to 57 dph, fish were offered a 1:1 (w/w) mixture of the weaning diet Caviar™ (Bernaqua, Belgium) and dried and powdered *Ulva lactuca*, which was produced at the IOLR (Eilat, Israel). After 57 dph, fish were fed only the starter feed Ranaan Dry feed (RDF, Israel) until the end of the trial at 79 dph (Table 2).

2.2 Trial 2: Comparison of the effect of water turbidity from live microalgae and white clay particles on larval and juvenile grey mullet performance

In order to test whether the advantage of water turbidity on larval performance is independent of turbidity source (live algae or clay), trial 2 compared the most effective turbidity level produced by live algae and white clay with a lower algae produced turbidity level as a control. The experimental treatments consisted of: (1) low water turbidity (0.8 ± 0.04 NTU; 0.25 x 10^6 cells of *N. oculata* mL^{-1}), (2) high water turbidity (1.2 ± 0.04 NTU; 0.50 x 10^6 cells of *N. oculata* mL^{-1}) and (3) high water turbidity (1.2 ± 0.02 NTU) produced by white clay, which was purchased from DAS Terracotta. F.I.L.O. (Pero, Italy). In this trial, grey mullet eggs, at gastrula stage, were stocked in twelve 1.5 m^3 V-tanks (100 eggs L^{-1}). The mullet larvae were exposed to the treatments from 2-30 dph while length was measured daily to 29 dph and survival at 50 dph. Larval rearing and husbandry conditions, as well as feeding protocols, were similar to those previously mentioned in trial 1.

2.3 Water turbidity assessment and control

All turbidity values in these trials were determined on triplicate water samples from each tank, including the control no algae treatment, which were first filtered (40 µm mesh size) before being read with a Turbidometer (Lovibond Turbi-check, Amesbury, England). The turbidity value for

Nanno B (1.20 NTU) was based on the current concentration of *N. oculata* ($0.5x10^6$ cells mL^{-1}) used at the IOLR for larval rearing. Previous studies carried out at the IOLR (unpublished data) demonstrated that levels above this concentration significantly reduced rotifer consumption in gilthead sea bream (*Sparus aurata*) larvae. *I. galbana* has a cell size of *ca.* 5 µm, while *N. oculata* is *ca*. 1.5 µm. The concentration of the larger *I. galbana* needed for achieving a turbidity value of *ca* 1.20 for Iso B was empirically determined. The turbidity values for Iso and Nanno A were based on half the *I. galbana* or *N. oculata* concentrations used to achieve the B turbidity values and were empirically determined. Microalgae and the clay were added twice daily during the morning (08:30) and afternoon (14:30) in the larval rearing tanks, before larval feeding, in order to maintain stable water turbidity levels. Algae in the rearing tanks were diluted due to constant water exchange. Consequently, the afternoon algal addition was based on measured turbidity levels, which were increased to designated values.

2.4 Determination of rotifer consumption, growth and survival in grey mullet larvae

In order to determine rotifer consumption, five larvae were sampled from each tank in the experimental system 90 min after feeding them with enriched rotifers. Larvae were then sacrificed, according to ethical standards, with an overdose of the anesthetic tricaine methanesulfonate (MS-222, Sigma-Aldrich, Rehovot, Israel) and fixed in 10% buffered formalin and stored at 4 ºC until counting the mastaxes (indigestible part of the rotifer's feeding apparatus) in the fish's digestive tract. Anecdotal observations determined that mastaxes can be expelled 2-3 h after feeding suggesting that 90 min would allow a good approximation of rotifer consumption.

Fish growth was measured as dry weight (DW) when the larvae were 15, 18 and 25 dph at the end of the turbidity Experiment 1 and at 29 dph at the end of Trials a and b in Experiment 2. This was

carried out by collecting *ca*. 130 larvae per tank, sacrificing them with an excess of MS-222 and then washing them with distilled water. Fish samples were then dried at 70 ºC for 24 h followed by their weighing (A&D HD-120 analytical balance, Japan).

Due the minute size and rapid deterioration of dead larvae, it was not possible to accurately follow mortality in large tanks during the course of the experiment. Consequently survival values were determined only at the end of both trials and were expressed as a percentage of the surviving fish, when the fish were harvested, over the number of the fertilized eggs initially stocked (adjusted for hatching rate and survival after 24 h) in tanks and taking into account the number of sampled fish for analytical purposes. Samples for measuring the digestive tract enzyme activities in the turbidity trial were sampled at hatching, 18, 25, 40, 61 and 79 dph.

2.4 Digestive enzyme activities

In trial 1, it was not possible to separate tail and trunk musculature from the abdominal region of whole larvae younger than 60 dph, while older fish (60 and 79 dph) were dissected to separate the pancreatic and intestinal segments. Digestive enzyme activities were determined using the following numbers of fish (3 replicates per experimental condition): 350-400 fish at 18 dph, 92-172 fish at 25 dph, 12 fish at 41 dph, 3-6 fish at 61 dph, and 4-6 fish at 79 dph. Dissection was conducted under a dissecting microscope on a pre-chilled glass plate maintained at 0 °C and lyophilized (FD-80, Boyikang, China) and shipped to IRTA's facilities for their analysis. For quantifying the activity of enzymes, lyophilized samples were homogenized (Ultra-Turrax T25 basic, IKA©-Werke, Germany) in 30 volumes (v/w) of mannitol (50 mM mannitol, 2 mM Tris-HCl buffer; pH 7.0), centrifuged and the supernatant removed for enzyme quantification. Then, 1 mL of the supernatant was stored at -20 ºC for leucine–alanine peptidase quantification and the rest of the homogenate was used for brush

163 border purification (Crane et al., 1979; Gisbert et al., 2018). Enzyme activities for pancreatic and
164 intestinal enzymes (U mg protein^{-1}) were determined as described in Gisbert et al. (2009) and
165 processed within 15 days to keep their activities intact (Solovyev and Gisbert, 2016). Trypsin and
166 chymotrypsin activities were assayed using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) and
167 BTEE (benzoyl tyrosine ethyl ester) as substrates (Holm et al., 1988; Worthington, 1991).
168 Chymotrypsin activity was only assayed in the 79 dph group, whereas for younger groups the activity
169 was below the detection limit levels of the assay. Alpha-amylase was measured using starch as
170 substrate (Métais and Bieth, 1968); bile salt-activated lipase activity was assayed using p-nitrophenyl
171 myristate as substrate (Iijima et al., 1998). Alkaline phosphatase activity was measured using 4-
172 nitrophenyl phosphate (Bessey et al., 1946); the assay of the cytosolic peptidase, leucine–alanine
173 aminopeptidase was performed using leucine-alanine as substrate (Nicholson and Kim, 1975).
174 Soluble protein of extracts was quantified by means of the Bradford's method (Bradford, 1976). All
175 the assays were made in triplicate from each pool of larvae (biological replicate) and absorbance read
176 using a spectrophotometer (Tecan^{TM} Infinite M200, Switzerland).

177

178 *2.5 Statistics*

179 Statistical analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad
180 Software, San Diego California USA, www.graphpad.com). All data are presented as mean ± SEM.
181 Outliers were identified by calculation of the Z value using the Grubbs test (Rousseeuw and Leroy
182 2003) and removed if calculated Z value was higher than the tabulated value. Every fish sampled for
183 mastax measurement was considered a treatment replicate (15-20 larvae from each age of 2-5 dph
184 were sampled per treatment). Percentage data values were first arcsine transformed, and then analyzed
185 by one-way ANOVA and Barlett's test for equal variances. If significance ($P < 0.05$) was found after
186 ANOVA analysis while Barlett's test was not significant ($P > 0.05$); then, testing differences between

groups was carried out by Newman-Keuls Multiple Comparison test. In cases where ANOVA and Barlett's test were both significant ($P < 0.05$); then, the non-parametric Kruskal Wallis Test was applied followed by Dunn's multiple Comparison test to determine significant ($P < 0.05$) differences among treatments. Regression data sets employed Akaike's Information Criteria (AIC) to compare linear, second order polynomial and other models to determine which most likely generated the data. The effect of water turbidity on the overall activity of pancreatic and intestinal enzymes was evaluated by Principal Component Analysis (PCA) in 79 dph fish (Statistica 7.0, StatSoft, Inc.).

2.6 Ethics statement

All animal experimental procedures were conducted in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

3. Results

3.1 Trial 1: the effect of water turbidity from two species of live microalgae on larval and juvenile grey mullet performance and ontogeny of the digestive tract functionality

Figure 1a shows that the water turbidity (NTU) values for different experimental conditions, Turbidity values of treatments using microalgae regardless of the microalgae and cell density used were significantly different from the control group (no algae) from 1 to 23 dph ($P < 0.05$) In Figure 1b the turbidity (NTU) values in trial 2 are shown comparing the Clay B treatment (1.2 NTU) with the low (0.75 NTU) Nanno A and high Nanno B (1.2 NTU) microalgae treatments. The turbidity values of the Nanno B and Clay B were very similar while both of these microalgae treatments were significantly ($P < 0.05$) different from those of Nanno A on each day from 2 to 24 dph.

Trial 1: rotifer consumption, larval survival and growth under the different turbidity conditions

Figure 2a demonstrated a significant ($P < 0.05$) water turbidity effect on rotifer consumption in grey mullet larvae aged 5 dph that was independent of the type of microalgae used for increasing water turbidity. Larvae in the Iso B and Nanno B treatments consumed significantly ($P < 0.05$) more rotifers than in the Nanno A and Control groups. The same trend was observed for the Iso A treatment, it but was not significant ($P > 0.05$). Interestingly, this pattern of rotifer consumption was very similar to that of fish survival much later on (Fig. 2b). Fish exposed to high microalgae water turbidity levels (Iso B, Nanno B) from 2 to 23 dph survived significantly ($P < 0.05$) better at 51 dph, which was about 4 weeks after the treatments had been discontinued, than fish feeding at the lower water turbidity values (Iso A, Nanno A) or in clear water (control). However, despite the effect of water turbidity on prey consumption and larval survival, there was no significant ($P > 0.05$) treatment effect on their growth performance (Fig. 2c).

Trial 1: digestive enzyme specific activity as a function of fish age, microalgae water turbidity levels and diet

Trial 1 results indicated that water turbidity treatments tested from 2 to 23 dph larvae had no obvious effects on the specific activities of digestive enzymes when they were measured between 18 and 79 dph (Figs. 3-5). On the other hand, the results from PCA (Fig. 6) suggested that lower water turbidity, regardless of the microalgae used, was correlated with higher pancreatic and intestinal enzyme activity in 79 dph grey mullet and much less so to the control and the high turbidity treatments. The exceptions of Nanno A and Nanno B samples showed intermediate values in comparison to the rest of the other groups.

231 Diet composition during the period of enzyme sampling did significantly ($P < 0.05$) affect the specific
232 activity of bile-salt activated lipase and alkaline protease. Bile salt-activated lipase decreased from
233 18 dph (Fig. 3a), when larvae were feeding on high lipid containing rotifers and *Artemia* metanauplii
234 (Table 2), to the lower dietary lipid levels of the co-feeding period based on Caviar: *U. lactuca* (1:1
235 w/w) diet from 25-57 dph (Table 2). However, after switching to the RDF diet at 57 dph, the increased
236 lipid of this feed (14%) resulted in a marked increase of the bile salt-activated lipase activity ($P <$
237 0.05). Similarly, dietary protein decreased from the high levels found in rotifers and *Artemia*
238 metanauplii (2 to 25 dph) (Table 2), which corresponded to high alkaline protease activity, to the
239 reduced protein levels of the Caviar: *U. lactuca* (1:1) diet fed between 25 and 57 dph with the
240 subsequent decrease in alkaline protease activity (Fig. 3b). However, the activity of these enzymes
241 tended to increase when fish were ingesting the higher protein levels of the RDF diet from 58 to 79
242 dph (Fig. 3a, b).

243 In contrast, trypsin activity was not affected by the diet throughout the entire sampling period (Fig.
244 3c; $P > 0.05$). The cytosolic enzyme leucine-alanine aminopeptidase (LAP) significantly ($P < 0.05$)
245 decreased in all microalgae treatments from 18 to 61 dph regardless of the live prey provided, but
246 then increased ($P < 0.05$) in 79 dph fish when the fish were fed the RDF diet (Fig. 3d). The activity
247 of the digestive tract marker for brush border membrane (BBM) development, alkaline phosphatase
248 (AP), significantly increased 8.0 times in fish from 40 to 79 dph (Fig. 4a; $P < 0.05$). Consequently,
249 the AP/LAP ratio, an indicator of gut maturation, in almost every treatment peaked at 61 dph, but
250 then declined in 79 dph fish (Fig. 4b).During the period between 40 to 79 dph, α-amylase specific
251 activity (Fig. 4c) increased 5.3 times independently of diet. The alkaline protease/lipase ratio values
252 showed higher protease activity over bile salt-activated lipase levels at 18 dph when larvae were
253 feeding on highly digestible rotifer and *Artemia* protein sources (Fig. 4d). However, total alkaline
254 protease activity dropped compared to bile salt-activated lipase activity at 25 dph, when the fish began

255 to feed on the Caviar: *U. lactuca* diet. On the other hand, the ratio increased significantly ($P < 0.05$)
256 from 25 to 61 dph. Moreover, the amylase/trypsin and amylase/protease ratios supported the
257 increasing capability of amylase production with age (Fig. 5a, b).

258

259 *3.2 Trial 2: larval rotifer consumption, growth and survival*

260 The effect of Nanno A, Nanno B and Clay B treatments on rotifer (mastax) consumption, DW in 30
261 dph larvae and survival in 50 dph fish and are shown, respectively, in Fig. 7a, b, c. The results from
262 trial 2 were in agreement with those of trial 1 and demonstrated that grey mullet larvae exposed to
263 the higher microalgae produced turbidity outperformed ($P<0.05$), in terms of food ingestion, growth
264 and survival, their cohorts reared under lower microalgae produced turbidity as well as larvae
265 exposed to the identical high turbidity level (1.2 NTU) produced from clay.

266

267 **4. Discussion**

268 The beneficial effects of the presence of microalgae in the rearing tanks of the larvae of many farmed
269 species have long been recognized (Naas et al., 1992; Reitan et al., 1997; Cahu et al., 1998; Lazo et
270 al., 2000; Skiftesvik et al., 2003; Faulk and Holt, 2005). In support of this, different authors have
271 reported the importance of microalgae addition in larval rearing tanks for several mullet species such
272 as the stripped grey mullet (Tamaru et al, 1994) and the thick lipped grey mullet, *Chelon labrosus*
273 (Ben Khemis et al., 2006). Various hypotheses have been postulated for explaining how microalgae
274 might benefit larvae, including (1) providing a direct supply of micronutrients (Van Der Meeren,
275 1991) that trigger key physiological processes (Hjelmeland et al., 1988), (2) releasing of appetite
276 stimulating components (Stottrup et al., 1995), and (3) influencing the bacterial composition of the
277 rearing water and consequently, the larval gut microbial flora (Skjermo and Vadstein, 1993; Bentzon-

278 Tilia et al., 2016). Apart from these potential advantages, a number of authors have also suggested
279 that microalgae turbidity creates a backlighting effect that would contrast the zooplankton prey
280 against their background facilitating larval foraging behavior and in particular, prey detection and
281 hunting success by the larvae (van der Meeren, 1991, Utne-Palm, 2002). In the present study, the
282 increased consumption of rotifers at the higher water turbidity levels (*ca* 1.20 NTU), independently
283 of the microalgae species used, appeared to suggest that the turbidity and the subsequent backlighting
284 effect of the microalgae was the main factor influencing rotifer ingestion and larval performance. In
285 Atlantic halibut, *Hippoglossus hippoglossus* (Naas et al., 1992) and stripped grey mullet (Tamaru et
286 al., 1994), it was suggested that the microalgae suspension enhanced visual contrast, allowing larvae
287 to better detect their prey. On the other hand, Rocha et al. (2008) argued that the larval prey capture
288 was influenced by both the effect of algae on water light conditions, as well as by the substances
289 provided by their presence, although the extent of this effect on fish larvae might be species-specific.
290 In fact, an equally plausible interpretation of the results from the first trial was that there are
291 microalgae unidentified factors that are common to both *I. galbana* and *N.oculata*, triggering key
292 physiological processes that modulate prey consumption and larval performance. This line of
293 reasoning is clearly reinforced by the result showing that clay, added to the tank at the same turbidity
294 as the most effective microalgae concentration (Nanno B), performed markedly less well, in terms of
295 prey ingestion, growth and survival. Overall, the present study suggests that in grey mullet larvae,
296 water turbidity played a more subordinate role compared to the likely contribution of microalgae
297 compounds that were released into the water and/or absorbed by the larval digestive tract. On the
298 other hand, the benefit of clay may be species specific and/or vary depending on the rearing
299 conditions. In Atlantic halibut larval rearing, the addition of inorganic clay during the first feeding
300 phase reduced opportunistic pathogenic bacteria such as *Vibrio* spp. in the tank water compared to
301 microalgae supplementation (Bjornsdottir et al., 2011). Similarly, clay addition reduced bacterial load
302 in the larval rearing tanks of cod (*Gadus morhua*) (Attramadal et al., 2012. In fact, the replacement

303 of microalgae in order to achieve so-called “environmental shading” with inorganic clay has become
304 the industrial standard in a number of halibut hatcheries in Norway. However, the negative effects
305 of very high turbidity can outstrip the benefit of microalgae or clay addition when fish are visual
306 feeders (Confer et al., 1978; Gregory and Northcote, 1993).
307 The addition of microalgae to larval rearing tanks has been shown to improve survival in a number
308 of species such as halibut (Naas et al., 1992), cod (van der Meeren et al., 2007), European sea bass,
309 *Dicentrarchus labrax* (Cahu et al., 1998) and cobia, *Rachycentron canadum* (Faulk and Holt, 2005).
310 Interestingly, the significant effect of live microalgae addition and water turbidity level on rotifer
311 consumption in 5 dph larvae was in agreement with the larval survival results measured in 51 dph
312 fish (28 days after the microalgae treatments had discontinued). This suggests that larval survival, to
313 a large extent, is determined early on during rotifer feeding. In fact, the onset of exogenous feeding
314 after the reabsorption of the yolk sac is a critical time during larval development that is frequently
315 characterized by massive mortality (Yufera and Darias, 2007) if young fish are not consuming
316 sufficient levels of prey such as rotifers. Normal neural development, gut maturation, muscle function
317 and growth can be compromised leading to mortality if there is any delay or reduction in first feeding
318 (Gisbert et al. 2004).
319 It is conceivable that larvae reared under the higher microalgae produced water turbidity were
320 surviving better as they were consuming more rotifers and digesting them more efficiently as gut
321 maturation would be accelerated. In support of this, the influence of green water on enhanced
322 pancreatic enzyme production and accelerated brush border membrane development and its
323 subsequent enzyme activity improved survival in European sea bass larvae (Cahu et al., 1998;
324 Zambonino-Infante and Cahu, 1994). However, in the present study no significant differences were
325 found in enzyme activity among larvae from the different turbidity treatments from 18 dph onwards.
326 This may have been due to sampling larvae after the beneficial effect of green water on the maturation
327 of the digestive system was no longer detectable. Cahu et al. (1998) described in European sea bass

that the effect of microalgae addition on the activity of digestive enzymes was only observed until 16 dph, but not in older fish.

In contrast, the PCA results of our study implied that 1 to 23 dph larvae exposed to a lower water turbidity of 0.76 NTU, independent of the microalgae species producing it, appeared to be associated with higher digestive enzyme activities in 79 dph juveniles than their cohorts in the control (0.26 NTU) and high turbidity (*ca*. 1.20 NTU) treatments. This suggests that relatively low levels of microalgae addition is necessary to stimulate digestive enzyme activity, whereas a higher microalgae concentration would be less stimulatory but potentially more effective to enhance other physiological pathways modulating survival and growth (e.g. stimulating the immune system). Taken altogether, the benefits of "greening" the larval rearing tanks on fish performance later on in development, likely includes a range of factors that are microalgae concentration and species specific dependent.

In order to analyze the ontogeny of digestive enzymes in 60 and 79 dph juveniles in this study, it was necessary to separate the tail and trunk musculature from the abdominal region in order to prevent the inclusion of excess protein in the sample homogenate. This would have led to underestimating digestive tract enzyme activities. On the other hand, dissecting out intestinal and pancreatic tissues in less than 60 dph larvae was technically not possible. Consequently, enzyme analyses on whole body larvae was done in all fish younger than 60 dph larvae. Nevertheless, this was considered acceptable as an estimation of intestinal and pancreatic enzyme activity as larval trunk and tail musculature contain considerably less protein than these tissues in juvenile fish.

The composition of the diets that grey mullet were consuming during development appeared to have influenced the specific activities of bile-salt activated lipase and total alkaline proteases. It should be noted that no acid protease activity was found in experimental samples (data not shown), regardless of the presence of a stomach (Oren, 1981). This suggested that protein digestion in grey mullet larvae

353 is mainly accomplished by alkaline proteases as found in other marine fish larvae and early juveniles
354 (Gisbert et al., 2009). *Ulva* spp. are a relatively rich source of starch (Korzen et al., 2016) where fish
355 α-amylase can hydrolyze the α-1, 4 glycoside bonds of this dietary component. This argues that the
356 increase in α-amylase activity from 25-61 dph, which was likely genetically directed, resulted in an
357 increasing ability to digest *U. lactuca* carbohydrate and potentially exposing more *Ulva* protein for
358 protease digestion. The maltose resulting from amylase digestion is absorbed as glucose after maltase
359 brush border activity. Conceivably, the catabolism of glucose as an energy source might be protein
360 sparing and therefore promote growth. The suggestion that the ontogeny of this carbohydrase is
361 genetically programmed is supported by similar high α-amylase activities found in grey mullet fry
362 that were weaned onto starch poor diets that were rich in fish meal or with a high level of fish meal
363 substitution by plant proteins (Zoutien et al., 2008; Gisbert et al., 2016).
364 In fact, the ontogeny of digestive enzyme activities in grey mullet larvae and juveniles, in general,
365 appeared to be more a function of age and genetic programming than dietary modulation. A case in
366 point are the two enzymes indicative of enterocyte development; (1) the brush border membrane
367 (BBM) alkaline phosphatase, which is a marker of nutrient absorption, and (2) the cytosol based
368 leucine-alanine peptidase (LAP), which is involved in protein intracellular digestion (pinocytosis) in
369 the cytosol of enterocytes. As the BBM develops together with increasing enzyme activity, there is a
370 parallel decrease of and dependence on intracellular digestion activity, resulting in an increase of the
371 AP/LAP ratio (Ma et al., 2005) until reaching the adult mode of digestion. The AP/LAP ratio abruptly
372 increased in *C. labrosus* at 8 dph and then significantly decreased to 36 dph. This prompted the
373 authors to conclude that gut maturation took place rapidly and early in this species. In contrast, the
374 present study on grey mullet showed that AP activity dramatically increased an average of *ca.* 8 fold
375 between 40 and 79 dph, where the peak AP/LAP ratio was detected at 61 dph. In fact, there was a
376 marked drop in the ratio at 79 dph caused by LAP levels abruptly increasing. Zouiten et al. (2008)
377 studying *C. labrosus* found a similar, but much earlier AP/LAP ratio pattern. These results argue for

a late maturation of the BBM and/or the transition from a carnivorous to an omnivorous/herbivorous mode of feeding as grey mullet juveniles swim to estuarine environments (Oren, 1981). In addition, the late increase in LAP levels may be due to the lack of acid proteases in grey mullet requiring the combination of both extracellular (intestinal lumen) and intracellular (enterocyte) digestion in order to process proteins more effectively.

The suggested late age of the carnivorous-herbivorous shift in grey mullet is supported by the steadily increasing activity of α-amylase from 25-79 dph, particularly between 40 to 79 dph. Alpha-amylase activity is much higher in herbivorous and omnivorous fish compared to carnivores (Solovyev et al., 2015). Overall, these results suggested that 61-79 dph grey mullet juveniles, which approximate the age of this species moving to estuaries (Gisbert et al., 2016), have the capacity to digest both protein and starch. This allows for the exploitation of the relatively starch rich microalgae (Zemke-White and Clements, 1999) and macroalgae (Horn et al., 1989) as well as benthic protein rich organisms characterizing these lower salinity estuarine waters (Oren, 1981). Taken one step further, the results broadly suggest that aquaculture feeds at this developmental stage should include not only considerable protein but also higher levels of starch or other low cost amylolytic energetic compounds compared to starter feeds fed to younger grey mullet or the juvenile stages of carnivorous species.

5. Conclusions

The results suggest that in grey mullet larvae, water turbidity played a more subordinate role improving fish performance than the contribution of microalgae chemical composition. On the other hand, microalgae supplementation during larval rearing did not have a conclusive effect on digestive tract enzyme ontogeny. Amylase activity steadily increased between 40 to 79 dph while the fish retained considerable protein digestion capability. This coupled with the late maturation of the gut at 61 dph argue for the capability of both carbohydrate and protein digestion as grey mullet juveniles transit from carnivory to omnivory while swimming to estuarine environments. This would allow for

403 the exploitation of the relatively starch rich microalgae and macroalgae as well as benthic, protein
404 rich organisms characterizing these waters.
405
406 **Acknowledgements**
407 This study was funded by the 7th Framework Program "Diversify- Exploring the biological and socio-
408 economic potential of new/emerging candidate fish species for the expansion of the European
409 aquaculture industry (project no. 603121)
410
411
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563 **Figure legends**

564 **Figure 1** Daily turbidity measurements (shortly before larval feeding) of the microalgae treatments
565 in (a) Experiment 1 and the (b) red and (c) white clay trials in Experiment 2. Different turbidity levels
566 (NTU) were significantly ($P<0.05$) different from each other in each experiment.

567 **Figure 2** The effect of microalgae turbidity treatments on larval (a) mastaxes consumed, (b) survival
568 at 51 dph and (c) dry weight (DW) at 15, 18 and 25 dph. Mastax consumption (5 dph) and percent
569 (%) survival (51 dph) values having different letter(s) were significantly ($P<0.05$) different.

570 **Figure 3** The effect of the control (C), Iso A (IA), Iso B (IB), Nanno A (NA), Nanno B (NB)
571 microalgae turbidity treatments on 25 dph larval (a) docosahexaenoic acid (DHA), eicosapentaenoic
572 acid (EPA) and arachidonic acid (ArA) levels (mg/g DW) as well as (b) saturated (SAT),
573 monounsaturated (MONO) and polyunsaturated fatty acid levels (mg/g DW). Values within an
574 essential fatty acid or fatty acid group having different letter(s) were significantly ($P<0.05$) different.

575 **Figure 4** The effect of the control (c), Iso A, Iso B, Nanno A, Nanno B microalgae turbidity treatments
576 on 23 dph larval (a) lipase and (b) alkaline proteases (c) trypsin and (d) leu-ala peptidase levels (U/
577 mg protein). Values within a turbidity treatment having different letter(s) were significantly ($P<0.05$)
578 different.

579 **Figure 5** The effect of the control, Iso A, Iso B, Nanno A, Nanno B microalgae turbidity treatments
580 on 23 dph larval (a) alkaline phosphatase (U/ mg protein), (b) alkaline phosphatase (P)/leu-ala ratio
581 (c) amylase (U/ mg protein) and (b) alkaline proteases/lipase ratio. Values within a turbidity treatment
582 having different letter(s) were significantly ($P<0.05$) different.

583 **Figure 6** The effect of the control, Iso A, Iso B, Nanno A, Nanno B microalgae turbidity treatments
584 on 23 dph larval (a) amylase/trypsin and (b) amylase/alkaline proteases ratio levels. Values within a
585 turbidity treatment having different letter(s) were significantly ($P<0.05$) different.

586 **Figure 7** The effect of the turbidity treatments; no algae (0), low turbidity *Isochrysis galbana* (ISO
587 A), high turbidity *Isochrysis galbana* spp. (ISO B), low *Nannochloropsis oculata* (NANNO A) and
588 high *Nannochloropsis oculata* (NANNO B) added to rearing tanks growing 2-23 dph grey mullet
589 larvae. PCA is based on the matrix of covariations that, in turn, was calculated on the specific activity
590 of all the studied (pancreatic and intestinal) digestive enzymes in 79 dph specimens.

591 **Figure 8** The effect of experiment 2 clay trials a (Nanno A, Nanno B and Clay B) and b (Nanno A2,
592 Nanno B2, Clay B2) on (a, b) mastax consumption, (c) larval survival (%) and (d) dry weight (DW).

Regression analysis of mastax consumption curves (selected from AIC analyses) demonstrated that the curve of the Nanno B treatment was significantly different ($P<0.05$) than the curves of Nanno A(2) and Clay B(2) in trials a and b. Survival (after arcsine transformation) and dry weight (DW) values within a trial having different numbers of asterisks (*) or letters were significantly ($P<0.05$) different.

Tables

Table 1 The microalgae treatments with their concentrations (cell mL^{-1}), designations and turbidities (NTU)

Treatments	**Designation**	**Turbidity (NTU)**
Control (no microalgae)	Control	0.26 ± 0.01^{a}
Isochrysis galbana A (0.0144×10^6 cell mL^{-1})	Iso A	0.77 ± 0.01^{b}
Nannochloropsis oculata A (0.2×10^6 cells mL^{-1})	Nanno A	0.75 ± 0.01^{b}
Isochrysis galbana A (0.0288×10^6 cell mL^{-1})	Iso B	1.18 ± 0.02^{c}
Nannochloropsis oculata A (0.4×10^6 cells mL^{-1})	Nanno B	1.20 ± 0.02^{c}

Table 2 Composition (% DW) of food used to feed the grey mullet at different stages of development

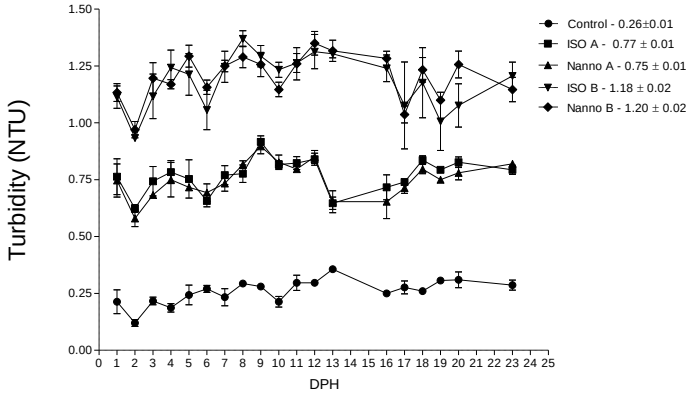
Feed	**Rotifers**[1]	**Unenriched *Artemia***	**Enriched *Artemia***	**Caviar™ (Bernaqua, Belgium)****	**Ranaan Dry Feed (RDF, Israel)***	***Ulva lactuca* (IOLR,Israel)**
Days fed (dph)	1-23	15	16-24	25-50	50-79	25-50
Protein (%)	57.3	49.1	53.1	55	56	34
Lipid (%)	27.3	12.8	28.7	15	14	7.4
Carbohydrate (%)	12.5	15	10	8	1	56
Ash	1.5	5	2	12	14.8	2.6

*800 µm

** Fed together with U. lactuca at a ratio of 1:1 (w/w)

Figures

(a)


Turbidity (NTU)
DPH
Control - 0.26±0.01
ISO A - 0.77 ± 0.01
Nanno A - 0.75 ± 0.01
ISO B - 1.18 ± 0.02
Nanno B - 1.20 ± 0.02

(b) **(c)**

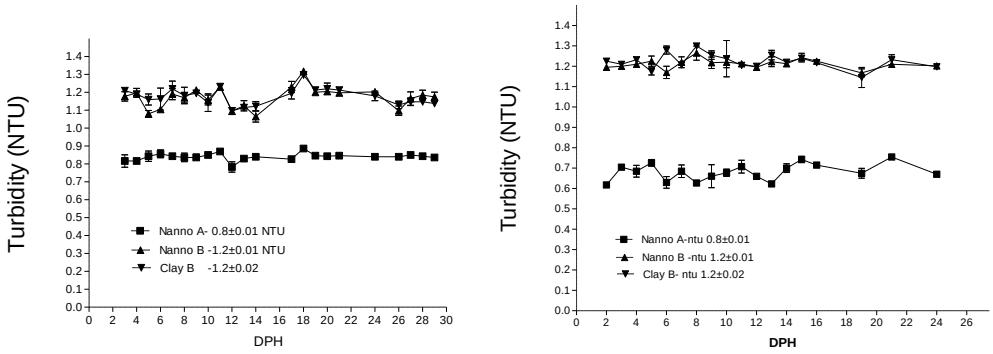
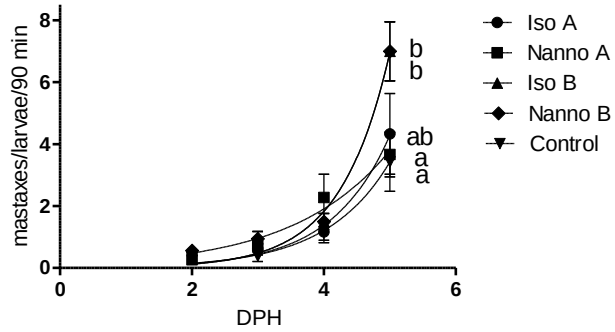

Turbidity (NTU)
DPH
Nanno A- 0.8±0.01 NTU
Nanno B -1.2±0.01 NTU
Clay B -1.2±0.02
Turbidity (NTU)
DPH
Nanno A-ntu 0.8±0.01
Nanno B -ntu 1.2±0.01
Clay B- ntu 1.2±0.02

Figure 1

(a)



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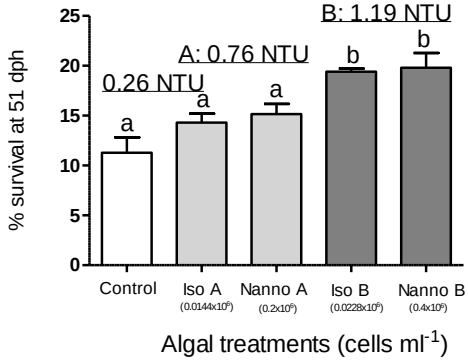
641 (b)



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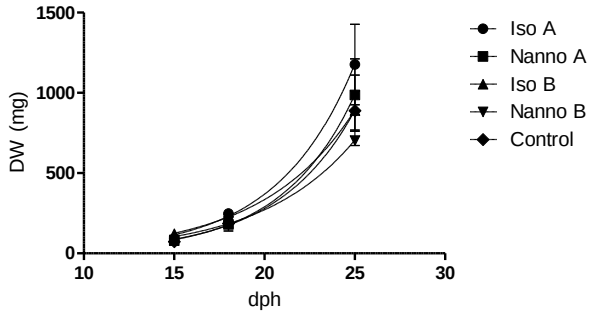
643 (c)



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645 **Figure 2**

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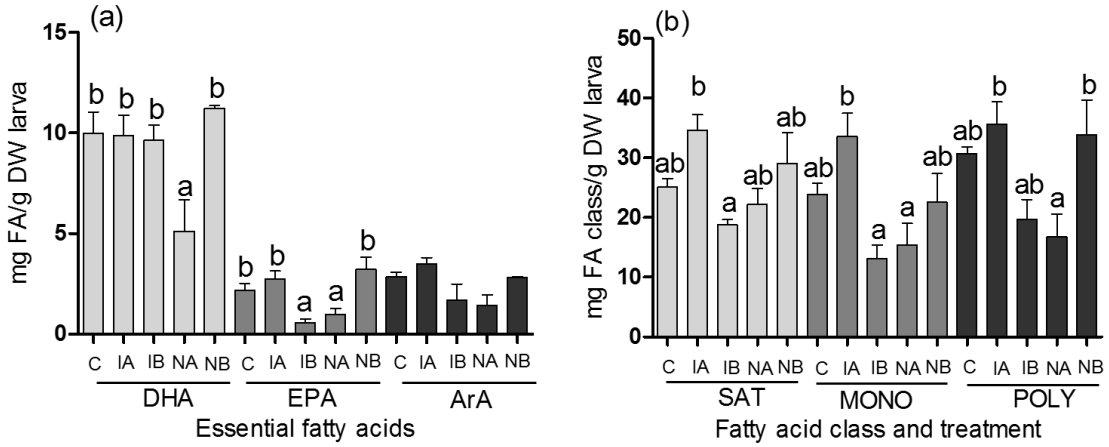

(a)
mg FA/g DW larva
15
10
5
0
b b b a b b b a a b b b a b
C IA IB NA NB C IA IB NA NB C IA IB NA NB
DHA
EPA
ArA
Essential fatty acids
(b)
mg FA class/g DW larva
50
40
30
20
10
0
ab b a ab ab b ab b a a ab ab b ab a b
C IA IB NA NB C IA IB NA NB C IA IB NA NB
SAT
MONO
POLY
Fatty acid class and treatment

Figure 3

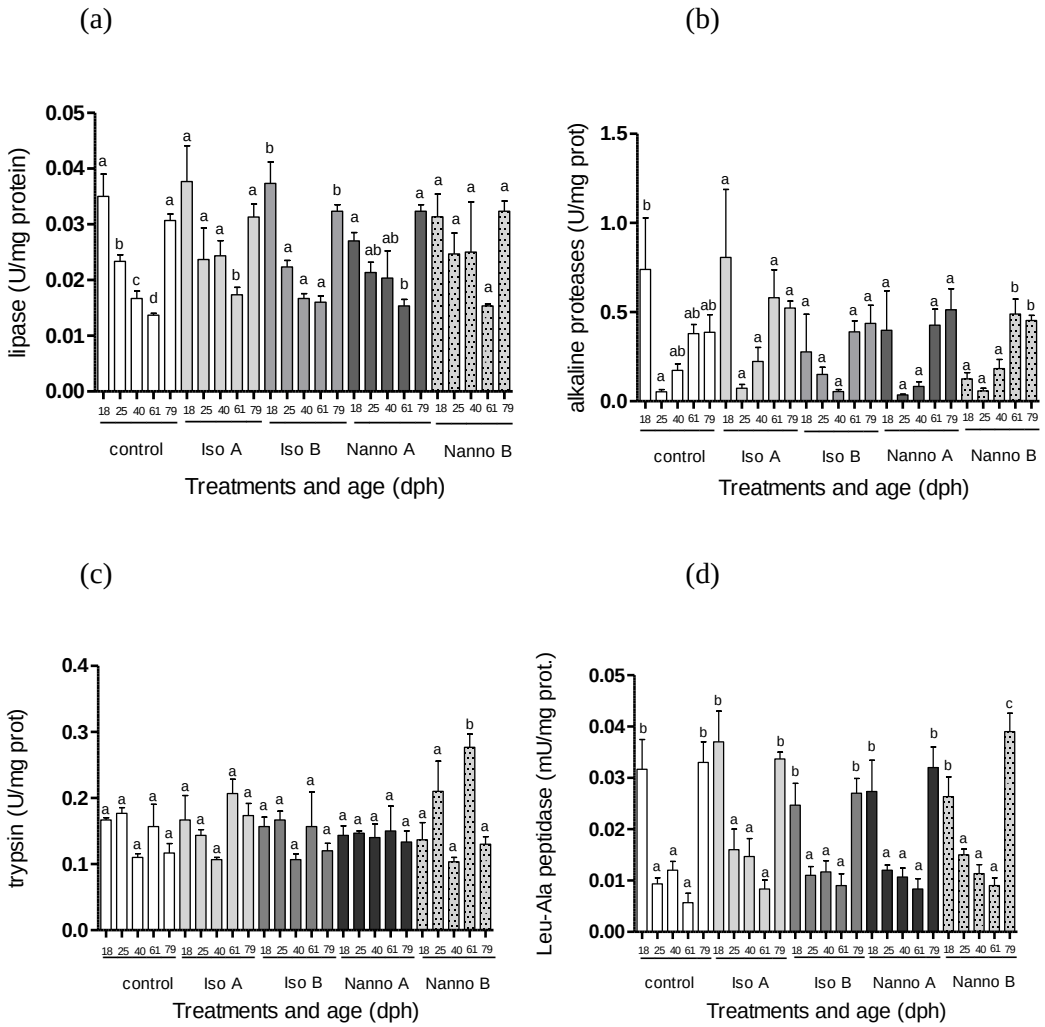
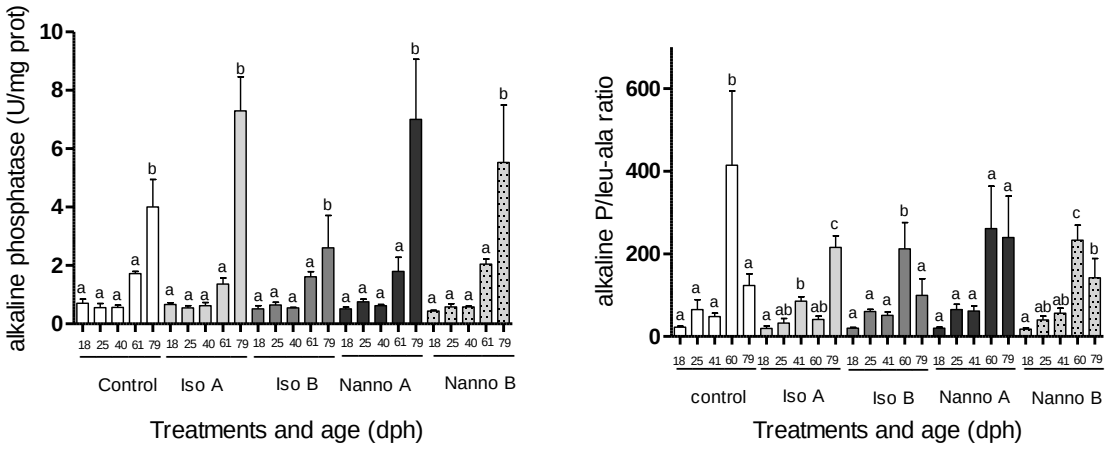

(a)
(b)
lipase (U/mg protein)
alkaline proteases (U/mg prot)
control
Iso A
Iso B
Nanno A
Nanno B
Treatments and age (dph)
(c)
(d)
trypsin (U/mg prot)
Leu-Ala peptidase (mU/mg prot.)

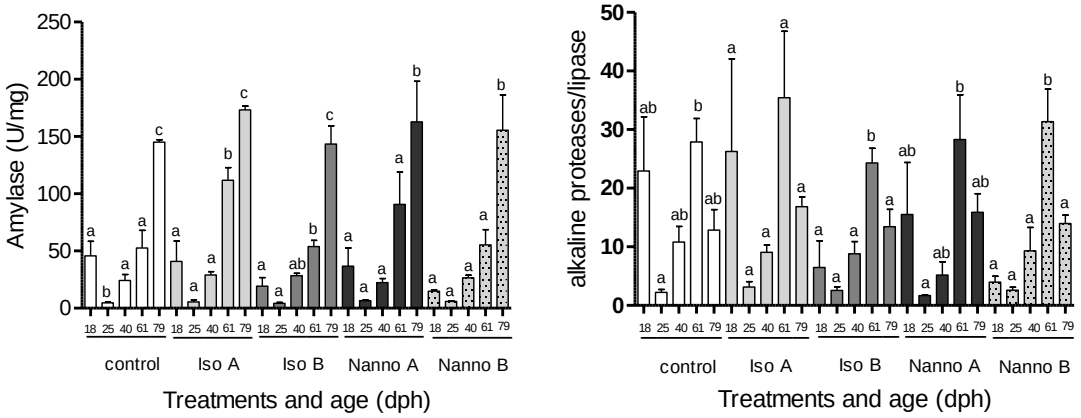
Figure 4

675 (a) (b)



676

677 (c) (d)



678

679 **Figure 5**

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(a) (b)

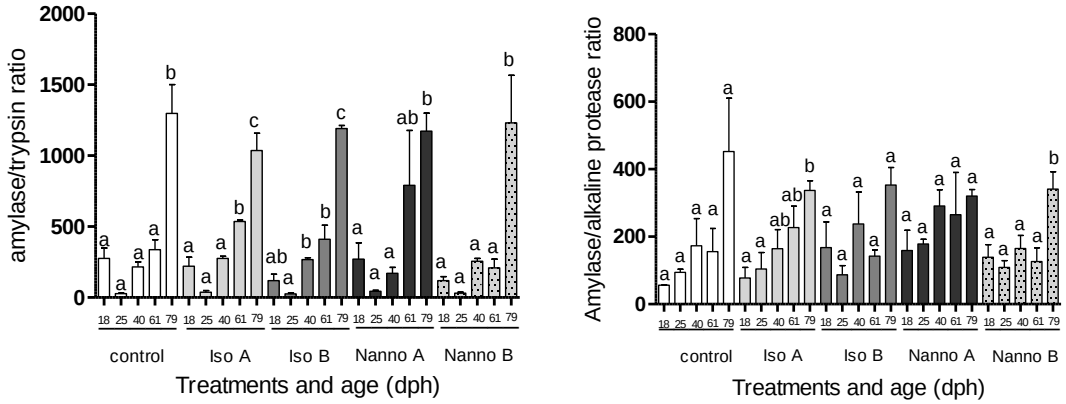
amylase/trypsin ratio
2000
1500
1000
500
0
Amylase/alkaline protease ratio
800
600
400
200
0
18 25 40 61 79
control
Iso A
Iso B
Nanno A
Nanno B
Treatments and age (dph)

Figure 6

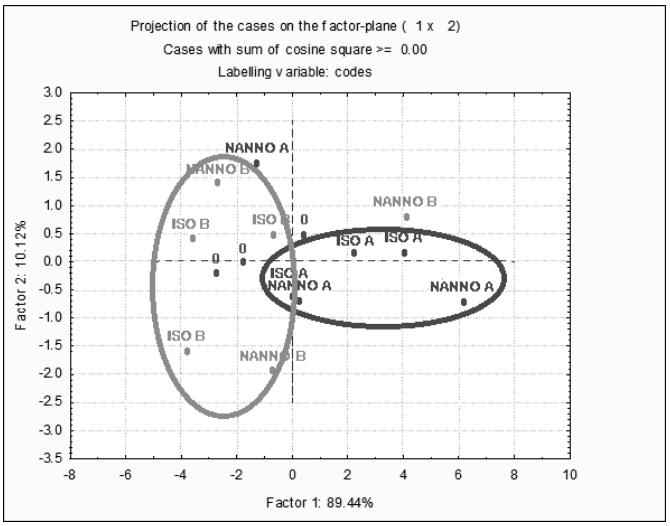
Projection of the cases on the factor-plane (1 x 2)
Cases with sum of cosine square >= 0.00
Labelling variable: codes
Factor 2: 10.12%
Factor 1: 89.44%
NANNO A
NANNO B
ISO B
ISO A
NANNO A
NANNO B
ISO A
ISO A
NANNO A
ISO B
NANNO B

Figure 7

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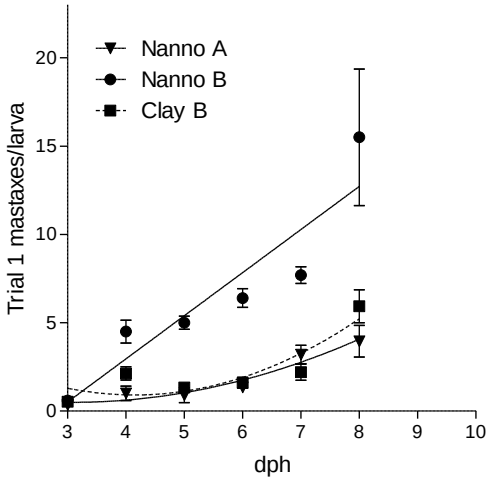
720 (a) (b)



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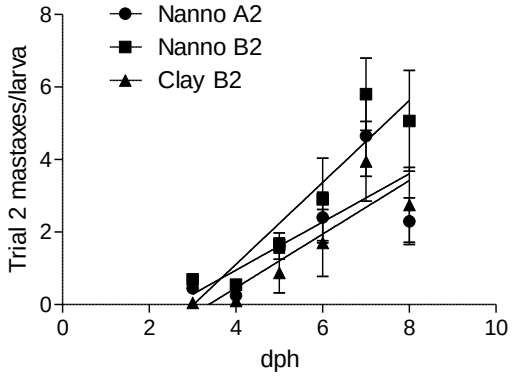
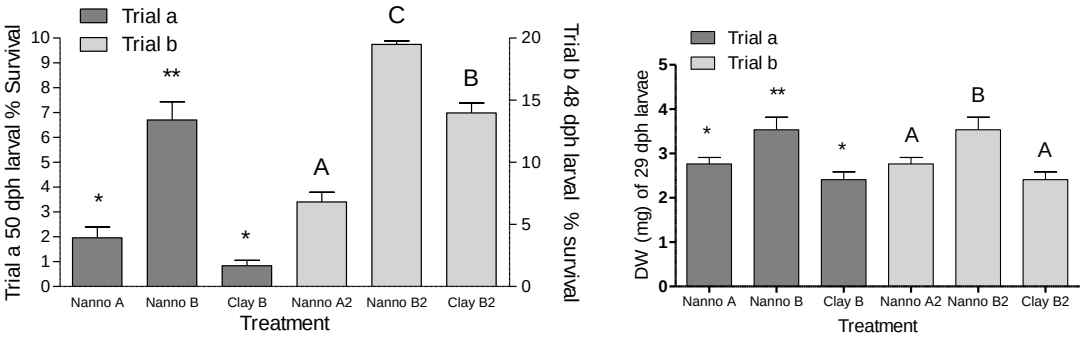


722

723 (c) (d)



724

725 **Figure 8**