Towards the standardization of brush border purification and intestinal alkaline phosphatase quantification in fish with notes on other digestive enzymes

Enric Gisbert[1*], Hector Nolasco[2], Mikhail Solovyev[3,4]

[1] Institut de Recerca i Tecnologia Agroalimentaries, Centre de Sant Carles de la Ràpita (IRTA-SCR), Crta. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Spain

[2] Centro de Investigaciones Biológicas del Noroeste, S.C., La Paz, B.C.S., México, 23090.

[3] Institute of Systematics and Ecology of Animals Siberian Branch of Russian Academy of Sciences, 11 Frunze St., Novosibirsk, 630091, Russia.

[4] Tomsk State University, 36 Lenin Ave., Tomsk, 634050, Russia

*Corresponding author: Tel: +34 977745427, Fax: +34 977744138; e-mail: enric.gisbert@irta.cat

Abstract

Assessing the activity of digestive enzymes is a common procedure in many biological, physiological and nutritional studies. After reviewing the available literature on fish digestive tract maturation and enzymatic activity (pancreatic and intestinal enzymes) published between 1994 and 2017, authors detected some possible methodological and/or interpretative inconsistencies in this kind of studies, and concluded that special attention should be paid on: *i*) the time of conservation of frozen samples prior their analysis, *ii*) the proper purification of the brush border of enterocytes by a double centrifugation step (Crane et al., 1979) when authors want to evaluate the activity of intestinal brush border enzymes in order to avoid the overestimation, particularly of alkaline phosphatase (AP), because it is present in other tissues; *iii*) the use of the proper reaction conditions at the normal range of values in terms of ions, temperature and intestinal alkalinity for the species of interest, and AP unit calculation. The implementation of these recommendations will promote the standardization of actual analytical procedures, as well as improve the reliability of comparative studies between different fish species or rearing procedures.

Keywords: brush border; enterocytes; alkaline phosphatase; spectrophotometry;

1. The relevance of assessing the activity of digestive enzymes

Assessing the activity of digestive enzymes is a common procedure in many biological, physiological and nutritional studies. In particular, evaluating the activity of digestive enzymes in nutritional studies is widely used when researchers aim to evaluate the digestive competence of a fish throughout its ontogeny in order to adapt feeding and rearing protocols to its digestive capacities (Lazo et al., 2011). These analyses are also commonly used for assessing the impact of a certain type of diet or nutrient on the functionality and/or maturation of digestive organs

(Zambonino-Infante and Cahu, 2007). This is of special relevance since the main role of the digestive system is to reduce food to very simple molecules (intestinal absorbable molecules) that are transported across the intestinal epithelium into the blood (Rønnestad et al., 2013). Thus, the rate of digestion in the digestive system limits the uptake of nutrients to the circulatory system and can potentially limit the growth of the whole organism, which is of special importance during larval development, when substantial changes in the structure, physiology, size, and body shape are produced in fish (Pittman et al., 2013).

In addition, the assessment of digestive enzymes, mainly pancreatic (alkaline proteases, lipase-like and carbohydrases), which are synthetized in the pancreas but act in the pyloric caeca and intestinal region, as well as gastric enzymes like pepsin (acid protease), may be used in *in vitro* digestibility studies in order to evaluate the capacity of fish to hydrolyse different feed ingredients of a certain diet (Dimes and Haard, 1994). These approaches by pH-stat and pH-shift (Alarcón et al., 2002) or the permeable membrane digestion cell system (Moyano et al,. 2015) are considered as the most useful method for understanding the digestibility of a diet or feed ingredient in fish, as these kind of analyses provide a fast and low cost effective alternative to *in vivo* trials when a large number of fish and methodological replicates are needed.

The last but not the least, digestive enzyme activities in fishes are indicative of their feeding ecology and trophic niche in natural conditions, thus, correlating well with their diet (Solovyev et al., 2014). Generally, herbivorous, omnivorous and detritivorous fishes may have higher carbohydrase activity (*i.e.* α-amylase) in relation to carnivorous ones, which have higher protease activity; thus, a higher carbohydrase/protease ratio has been proposed as a typical of herbivorous species, whereas this ratio has lower values in carnivorous ones (Hidalgo et al., 1999; Falcón-Hidalgo et al., 2011), even considering that animal nutritive polysaccharides (*i.e.* glycogen) are also digested by carbohydrases. Assessing fish digestive activities by measuring the activity of pancreatic digestive enzymes was also proposed by some authors as a good tool for discriminating between generalistic and/or opportunistic feeding strategies (Horn et al., 2006; German et al., 2009).

70 Assessing the activity of digestive enzymes are generally conducted by means of
71 spectrophotometric or fluorometric procedures, whereas some authors also use zymograms in
72 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for identifying and
73 qualitatively detect the presence of different types of enzymes or isoenzymes in crude extracts
74 (Santigosa et al., 2008; Falcón-Hidalgo et al., 2011). Spectrophotometric and fluorometric methods
75 for enzyme quantification are based on the incubation in a working solution (buffer) of a specific
76 substrate with an extract containing the enzyme or mixture of enzymes of interest that results in the
77 formation of the enzyme complex and the hydrolysis of the substrate. Substrate's hydrolysis is
78 dependent on enzyme activity and it is measured by changes in the absorbance or fluorescence of
79 the working solution (Bergmeyer, 2012). However, this review is not focussed on comparing both
80 procedures (spectrophotometry *vs* fluorimetry), since this issue has been already assessed and
81 review by previous authors (Rotllant et al., 2008).

82 Regardless of the particular and extensive use of these analytical procedures, the authors of
83 this contribution, while checking the available literature, have detected some common
84 inconsistencies in the preparation of enzymatic crude extracts, determination and/or description of
85 certain digestive enzyme activities, particularly alkaline phosphatase, that may affect the real
86 quantification and interpretation of the results derived from those studies. These issues will be
87 presented and recommendations provided.

88

89 **2. Optimal storage time of samples before enzyme activity analyses**

90 A potential underestimation when assessing the activity of digestive enzymes may be the long
91 storage of samples prior to their analysis, as well as their handling during bench work. In this sense,
92 very few studies provide detailed information about the time lapse between sample acquisition and
93 its processing, whereas this is of special relevance as it has been recently shown (Solovyev and
94 Gisbert, 2016). The former authors recommended to process samples for digestive enzyme analyses
95 as soon as possible, and do not conserve crude homogenates for assaying pancreatic (*e.g.* trypsin,

chymotrypsin, total alkaline proteases and α-amylase) and intestinal cytosolic (leucine-alanine peptidase, LAP) enzymes for more than 140 – 270 days at -20 ºC, since enzyme activity is generally reduced by more than a half of its original activity (freshly prepared crude homogenate). In addition, the former authors also reported a significant decrease in activity in the above-mentioned enzymes after sample (crude homogenate) handling for 5 h on ice (0–4 ºC). This loss of enzyme activity may be explained by the fact that the crude extracts contain relatively high amounts of different types of proteases that might lose their activity over time at low temperatures, presumably due to their auto- and heterocatalytic proteolytic degradation, but also due to the fact that these enzymes are still active even al low temperatures (-20 ºC), so they can damage other proteins, including enzymes, present in the crude homogenate. For instance, *in vitro* studies have reported that AP exposed to trypsin (10% w/w) showed 20% loss of activity after 30 min (Roberts and Chlebowski, 1984). On the contrary, purified enzymes demonstrate a great thermal stability at different temperatures. In the case of intestinal brush border enzymes, the purification of brush border (BB) membranes (Crane et al., 1979) allows removing another group of proteolytic enzymes (cytosolic and pancreatic) that are contained in the homogenate and can potentially hydrolyse and damage the BB enzymes (*i.e.*, alkaline phosphatase, maltase, aminopeptidase- N, among others). Finally, freezing and thawing cycles also affected enzyme activity in crude homogenates; thus, the activity of pancreatic enzymes decreases by a half after 1 to 2 freezing and thawing cycles, whereas that of BB intestinal enzymes is more stable (activity does not decrease after 5 freezing and thawing cycles). Considering the above-mentioned results, authors recommend including in each published study the time elapsed between sample collection, crude homogenate preparation and analysis, as well as reduce the bench-time of crude homogenates in ice (0–4 ºC) during daily operations, and aliquot crude homogenates after their preparation in order to avoid freezing and thawing cycles that may damage enzyme activity. These aspects should be considered when measuring other enzymes, whether they are digestive or not.

In addition to the above-mentioned factors, the potential impact on enzyme activities of other factors related to sample collection, *e.g.* freezing conditions (liquid nitrogen *vs* ice), potential

interference with other body tissues or parts of the digestive tract (whole body *vs* dissected organs) and/or faeces, should not be neglected, even though there is not available data on these issues.

3. Assessing the level of intestinal maturation: brush border purification and intestinal alkaline phosphatase determination

3.1 Brush border purification from the intestinal mucosa

The development of a full functional intestine implies different maturational and morphological events that are very well preserved among vertebrates. In particular, the appearance of a functional microvillus membrane in enterocytes constitutes a crucial step during larval development of fish for the acquisition of an adult mode of digestion (Zambonino-Infante and Cahu, 2001). The temporal coincidence between the rise of BB enzyme activities and the decline of cytosolic enzyme activities reveals an ontogenic process and characterizes the normal maturation of the enterocytes in developing fish larva. Thus, the activity of alkaline phosphatase (AP) from the intestine has been generally considered as a marker of gut maturation when activity values of this BB enzyme are reported as a ratio with the intestinal cytosolic enzyme LAP. Regardless of the popularity of this assay and its general utilization, we have found that AP activity determination is not properly conducted in a considerable number of studies. In this regard, authors decided to explore this issue by means of a bibliographic search using the Web of Science Core Collection™ (www.webofknowledge.com; Thomson Reuters™). The following search chain "alkaline phosphatase *AND* fish larvae" was selected to evaluate the methodology used for BB purification in the literature, resulting in a total of 113 published articles from 1994 to 2017. After removing the review-type articles, and those studies dealing with the quantification of AP by means of molecular biology (quantitative PCR) or histochemical procedures, or assessing its activity in the serum of fish (AP may be used as a biochemical marker for liver malfunction), authors used a final database

of 81 articles for their review (Supplementary file 1). These articles were inspected to identify which methodology was used for preparing the BB crude extracts used for AP quantification.

Results indicated that the purification of BB membranes in samples from whole larvae or dissected intestines (pool of individuals), that were posteriorly used for AP quantification, was properly done in 41 of the 81 consulted studies (50.6%), whereas the others had some methodological inconsistencies that did not allow the proper purification of BB membranes from the intestinal mucosa. Unfortunately, some authors apparently do not properly purify the BB membrane of enterocytes from the intestinal segment by at least a series of two centrifugation steps as described by Crane et al. (1979). The above protocol is based on a two-step centrifugation process, a first step at low RCF, *i.e*. 9,000 x *g* for 10 min at 4 ºC centrifugation, that allows the elimination of cell debris from tissue homogenates and AP originated from other tissues, followed by a high RCF centrifugation step of the supernatant (*i.e.* >30,000 x *g* for 20-25 min at 4ºC), in order to precipitate the BB membrane of enterocytes. However, 49.4% of the published studies used a protocol with just a single centrifugation step with centrifugation forces ranging from 5,000 to 16,000 x *g* for 5 to 10 min at 4 ºC (Table 1). The main problem with the above-mentioned approach is that BB membranes are not purified; then, AP could be from different origin, because it is widely found in the organism; consequently, activity values of AP may be overestimated, especially when analyses are conducted in whole specimens rather than in dissected guts. For instance, there are at least three distinct forms of AP in humans (placental, intestinal, and liver/bone/kidney) with specific patterns of tissue distribution, but not only restricted to the above-mentioned tissues (Weiss et al., 1988); whereas liver and kidney AP isoenzymes in fish are different from those from the intestine (Yora and Sakagishi, 1986). In addition, AP (specific or non-specific AP) is commonly used as a marker of cell differentiation in *in vitro* and *in vivo* studies in different tissues (Asakura et al., 2001; Goldsmith et al., 2002). According to Crane et al. (1979), the AP specific activity (U/mg protein) of the purified BB extract has to be close to 10 times higher than in whole intestine extract, an extract that is generally used for assessing the activity of intestinal cytosolic enzymes or secreted pancreatic enzymes; thus, checking the efficiency of BB purification is recommended.

In addition, the activity of any digestive enzyme, including AP, depends on the concentration and the turnover number of the enzyme (the maximum number of chemical conversions of substrate molecules per second that a single catalytic site will execute for a given enzyme concentration) (Bergmeyer, 2012). Thus, the different isoenzymes of AP obtained from different tissues may have different turnover rates; thus, hydrolysing the substrate at different rates, resulting in different activities per mol of enzyme and, which do alter the activity of AP in an unpredictable way. The same may be postulated for different fish species or stages of development, so this fact should be also taken into account when comparing the same species at different ontogenetic stages, because the isoenzyme' profile of the organism may also change, and different isoforms may also have different activity features like different *Km*, optimal salt concentration (NaCl), pH, temperature, divalent ions requirements, etc.

In conclusion, considering that the gut is not the main tissue in mass in the developing organism, the participation of AP from other developing tissues like bones, liver, kidney, cartilage, musculature or the nervous system do overestimate the real activity of AP from enterocytes (Yora and Sakagishi, 1986; Matusiewicz and Dabrowski, 1996; Shioi et al., 2002; Grotmol et al., 2005), and consequently, affecting the reliability of the AP/LAP ratio and its proper interpretation. Thus, it is strongly recommended to purify the BB of enterocytes from the intestinal mucosa according to the method described by Crane et al. (1979) when enzymes like AP, maltase or aminopeptidase-N want to be analysed in this intestinal cell compartment. It should be also considered that when fish larvae are too small to be dissected in order to remove the intestine from the whole specimen, BB purification is conducted from a pool of whole specimens.

Considering the above-mentioned results, authors recommend the following protocol described in Figure 1 for proper BB purification from enterocytes. However, the use of dithiothreitol (DTT) for preserving frozen BB enzymes (Solovyev and Gisbert, 2016) should be avoided, because of the inhibitor effect of DTT as a reducing agent on the activity of enzymes, including AP (Smith et al., 1996; Williams et al., 1999, Rong-Quim et al., 2000; Zappa et al.,

2001). Thus, as Crane et al. (1979) reported, purified BB membranes should be conserved in the same buffer used for tissue homogenization (Figure 1).

3.2 Determination of alkaline phosphatase activity

Authors have also revised the methodology for digestive AP quantification reported in the literature. In particular, the methods applied to quantify intestinal AP activity were revised in the 81 studies retrieved from the bibliographic (Table 2). The most common protocol for assessing the activity of intestinal AP in fish (63.0% of the studies) is the one proposed by Bessey et al. (1946), whereas 16.1% of authors used available commercial kits (*e.g.* Thermo Electron ALP Kit - Thermo Fisher Scientific, AP kit Sigma-Aldrich, AKP kit, Jiancheng, China). Other methodologies have been also used, but less frequently (≤5%) as shown in Table 2. Regardless of the method used, authors found some relevant inaccuracies in AP determination that need to be addressed.

Firstly, there is a great variability in the reaction buffers used by different authors as shown in Table 3 (commercial kits used for analysing the activity of AP were not considered). Thus, 64.2% of the studies used sodium carbonate (pH = 9.8-10.8), 7.4% glycine-NaOH (pH = 10.1-10.4), 3.7% diethanolamine (pH = 9.8) and 9.9% ammonium bicarbonate (pH = 7.8), magnesium acetate (pH value not indicated), borate (pH = 8.0), ringer solution (pH = 7.4) and/or distilled and seawater as reaction buffers for AP determination. The above-mentioned buffers may be considered adequate according to the working pH; however, the pH used to measure AP activity was not justified in many cases. It is obvious that pH affects the activity of AP, which must be measured under alkaline conditions typical of enterocytes; however, it seems doubtful that this cell membrane-bound enzyme that is in contact with the intestinal lumen reached high alkaline pH (pH > 9.0) values under normal conditions (Rønnestad and Morais, 2008; Solovyev et al., 2017). Thus, AP activity measured under highly alkaline conditions might not reflect the real activity of this brush border enzyme. Thus, it is advisable to measure AP at pH values of 8.0-8.6, *i.e.* using a TRIS-HCl, 50 mM buffer (McComb et al., 1979; Hethey et al., 2002).

Not only the reaction buffer used for AP determination showed some differences between studies, the inclusion of different divalent ions (*i.e.* magnesium, zinc) and chelating agents (*i.e.* EDTA or HEDTA) at different levels varied depending on the study considered. For instance, 81.2% of the studies (excluding commercial kits) used Mg^{2+} in the reaction mixture (*i.e.*, $MgCl_2$ used at concentrations ranging from 0.05 to 100 mM); whereas 2.9% of the works used magnesium acetate ($Mg(CH_3COO)_2$) at concentrations ranging from 3 to 2,000 μM (Table 3). In contrast, 18.8% of the studies did not use magnesium in the reaction mixture or did not indicate whether or not authors used it. Regarding the inclusion of Zn^{2+} in the reaction mixture, only 1.7% of the studies included it (Table 3). Thus, regardless of the use of divalent ions as potential activators of AP, it is recommended to first evaluate if there actually exists this dependence and in case of an affirmative answer, then test at which concentration divalent ions are required. Whenever it is possible, the optimal physiological concentration of zinc in the mixture should also be considered. Regarding the use of chelating agents, only 1.7% of the revised studies included them, whereas the rest of the studies did not use them or informed whether they used them or not. The use of chelating agents is questionable, since they are normally included, according to the authors, to protect the enzyme from potential toxic ions. However, if what is desired is to measure the AP activity in a sample, this needs to be measured with the ions that the sample (crude enzyme homogenate) contains allowing to evaluate the impact of experimental conditions on AP activity. In addition, if magnesium ions are included, adding chelating agents (even at the same concentration that of the divalent ion) is contradictory, since at least a significant part of the magnesium ions will be chelated and will not be available in the reaction mixture for "activating" AP.

There also existed a remarkable variability in the temperature at which AP was determined among the consulted studies (Table 3). In particular, 1.4% of the studies evaluated AP activity at 20 ºC, 5.8% at 25 ºC, 3.0% at 30 ºC, and 71.0% at 37 ºC, whereas 18.8% of the studies did not indicate the reaction temperature at which their AP measurements were performed. This analysis indicates that most of the assays for measuring AP activity were conducted following protocols developed for homoeothermic organisms with body temperatures ranging from 37 to 38 ºC, a range of temperatures really unusual for fish. Therefore, it is strongly recommended that the activity of

AP, and other enzymes, should be determined not even at the optimum temperature determined by *in vitro* studies, but at the physiological temperature in which the fish develops according to its temperature *preferendum*. A temperature of 25 ºC in the reaction mixture may be the most appropriate for most fish species inhabiting temperate waters.

Finally, differences in the wavelength (λ) used to measure the absorbance of released p-nitrophenol (pNP) from p-nitrophenyl phosphate (pNPP) substrate were also detected among studies (Table 3). Thus, among the studies that presented data on the wavelength at which AP was measured (excluding studies using commercial kits), 15.9% of them measured changes in absorbance at λ = 405 nm, 37.7% at λ = 407 nm, 1.5% at λ = 410 nm and 1.5% at λ = 420 nm, whereas in 29.0% of the examined cases do not specifically indicate the wavelength used (λ = 400-420 nm) and just referred to the original protocol (Bessey et al., 1946). In this sense, the former authors just recommended a range of wavelengths at which AP might be measured (λ = 400-420 nm). Considering that the absorbance of pNP is affected by the wavelength, it is recommended to use a wavelength of 405 nm as Walter and Schutt (1974) pointed out. In addition, different studies used different molar extinction coefficients for pNP: 6.7% used 18,300/M/cm (λ = 407 nm), 5.3% used 18,500/M/cm (λ = 405 nm) and 1.3% used 18,800/M/cm (λ = 405 nm), whereas the remainder studies (86.7%) used a standard curve. However, the use of a standard pNP curve is indicated in the text only in 19% of the cases, whereas it is assumed that authors used a standard curve according to the cited reference in the rest of the studies. As the molar extinction coefficient used to measure the released pNP by the hydrolysis of the substrate by AP affects the calculation of the enzyme units; it is advisable to use a molar extinction coefficient of 18,000/M/cm. In addition, it is strongly recommended, when using any molar extinction coefficients, that authors indicate that they have adjusted the absorbance at 1cm of light path. However, in order to eliminate potential errors related to avoiding the use of the path length of the sample for proper calculation of the molar extinction coefficient, it is advisable to construct a standard curve for pNP (λ = 405 nm *vs* μmoles of pNP in the reaction mixture) at the same experimental conditions at which AP activity will be measured, and also the use of the international enzyme unit definition for AP activity (1 AP Unit = μmol of pNP released per min). Considering the above-mentioned results, authors recommend to determine

AP in fish by means of a microplate kinetic method (λ =405 nm, readings every 30 seconds during 3-5 min) at 25 ºC, using p-nitrophenyl phosphate substrate (1 mM), Tris-HCl buffer (50 mM, pH 8), with and without the presence of divalent ions (10 mM) in order to determine their requirement, and using a p-NP standard curve.

4. Scaling fish larvae development: the use of proper developmental units for comparative purposes

The activity of the digestive enzymes during the initial stages of development is affected by a number of different factors, and their levels are closely related to the state of maturation of the secreting digestive tissues, which show important variations with species, water temperature and rearing conditions (Lazo et al., 2011). However, many authors when describing the ontogeny of the digestive function in fish larvae present and compare their data, expressed in total or specific enzyme activities, with that available in the literature using chronological units like days post-hatching (dph) or days after fertilization (daf). However, even though this approach may be informative from a zootechnical point of view (*i.e.*, age at which a certain type of live prey or diet should be introduced in rearing tanks), it is not very accurate with regard to its use in developmental, physiological and/or nutritional studies. The development of fish, as poikilothermic organisms, depends on water temperature, so using chronological units is not really accurate when comparing fish reared at different water temperatures; this is especially evident when authors compare data on digestive enzyme activities (*i.e.* changes and/or peaks in activity) (Lazo et al., 2011) or anatomical features (Parichy et al., 2009) from fish larvae reared at different temperatures (cold *vs* temperate or warm waters). As an example, fish with a same chronological age may be substantially different in terms of their morphological and functional development of their digestive system (pancreas and stomach development and intestinal maturation), and by extension in terms of the development of the other body systems, when reared in cold (*i.e.* cod *Gadus morhua* at 6 ºC; Kvåle et al., 2007) or warm waters (*i.e*. common dentex *Dentex dentex* at 19 ºC; Gisbert et al., 2009). Similarly, taking into account that temperature also affects the general level of fish

311 metabolism, that is to say that at high temperatures the digestion process will be accelerated when
312 compared to fish reared at low temperatures; thus, the peak of enzyme activity will be different in
313 fish reared at different temperatures but sampled at the same time. In addition, the time of the day
314 for sampling must be considered in order to avoid the potential effect of the circadian rhythm on
315 digestive enzyme activities (Montoya et al., 2010).

316 However, temperature is not the only parameter affecting fish larval development, other
317 factors like egg size (Gisbert et al., 1999), feeding protocols (Zouiten et al., 2011), diet quality
318 (Cahu et al., 2004) among others may also affect fish larval development; thus, fish with the same
319 age but reared under different biotic and/or abiotic conditions may be substantially different from
320 another one, which supports the need of avoiding using chronological units for comparative
321 purposes related to fish larval development. For instance, it has been recently evidenced in *A.*
322 *regius* regardless of the rearing conditions (*e.g.* mesocosm, intensive production, larval density,
323 water temperature and/or feeding sequence), the functional development of the digestive system
324 assessed by the activity of alkaline and acid proteases was a well-conserved process that generally
325 occurred within the same range of body sizes independently of larval age (Solovyev et al., 2016).
326 Consequently, authors recommend to scale fish larval development using thermal units
327 (temperature degree days), and complement this information with data on larval size (standard or
328 total length) for each age considered and/or the stage of development by using externally visible
329 anatomical features of the specimen.

330

331 **5. Conclusions**

332 The friendly use of protocols for assessing the activity of digestive enzymes has led to its
333 generalized use in many areas of fish biology; however, this has resulted in some methodological
334 and/or interpretative inconsistencies that this contribution aimed to address in order to improve the
335 reliability of data presented and discussed in future studies in this field. Thus, special attention
336 should be paid on: *i*) the time of conservation of frozen samples prior their analysis, *ii*) the proper

purification of the brush border of enterocytes by a double centrifugation step (Crane et al., 1979) when authors want to evaluate the activity of intestinal BB enzymes in order to avoid the overestimation of this enzyme present in other tissues; *iii*) the use of the proper reaction conditions at the normal range of values in terms of ions, temperature and intestinal alkalinity for the species of interest, and AP unit calculation. The implementation of these recommendations will promote the standardization of actual analytical procedures, as well as improve the reliability of comparative studies between different fish species or rearing procedures.

Acknowledgements

This review was partially funded by the grant AGL2014-51839-C5-5-R from the Ministerio de Economía, Industria y Competitividad (MINECO) of the Spanish Government and the Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo CYTED, Network LARVAplus (ref. 117RT0521). H.N.S. stage at IRTA (Spain) was funded by the CONACYT (Mexico) by means of the grant number 613211.

References

Alarcón, F.J., Moyano, F.J., Díaz M., 2002. Evaluation of different protein sources for aquafeeds by an optimised pH-stat system. J. Sci. Food Agri. 82, 697-704.

Asakura, A., Rudnicki, M.A., Komaki, M. 2001. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation 68, 245-253.

Bergmeyer, H.U., 1965. Methods of Enzymatic Analysis. Academic Press, London.

Bessey, O.A., Lowry, O.H., Brock, M.J., 1946. Rapid coloric method for determination of alkaline phosphatase in five cubic millimeters of serum. J. Biol. Chem. 164, 321–329.

360 Bramley, T.A. 1974. Treatment of immature mice with gonadotrophins. Effects on some enzymatic
361 activities of unfractionated ovarian homogenates. Biochem. J. 140, 451-460.

362 Cahu, C., Rønnestad, I., Grangier, V., Zambonino- Infante, J.L. 2004. Expression and activities of
363 pancreatic enzymes in developing sea bass larvae (*Dicentrarchus labrax*) in relation to intact
364 and hydrolyzed dietary protein; involvement of cholecystokinin. Aquaculture 238, 295-308.

365 Crane, R. K., Boge, G., Rigal, A. 1979. Isolation of brush border membranes in vesicular form from
366 the intestinal spiral valve of the small dogfish (*Scyliorhinus canicula*). Biochim. Biophys. Acta,
367 554, 264-267.

368 Dimes, L.E., Haard, N.F. 1994. Estimation of protein digestibility-I. Development of an *in vitro*
369 method for estimating protein digestibility in salmonids. Comp. Biochem. Physiol. 108A, 349-
370 362.

371 Falcón-Hidalgo, B., Forrellat-Barrios, A., Farnós, O.C., Hernández, K.U., 2011. Digestive enzymes
372 of two freshwater fishes (*Limia vittata* and *Gambusia punctata*) with different dietary
373 preferences at three developmental stages. Comp. Biochem. Physiol. 158B, 136-141.

374 Garen, A., Levinthal, C. 1960. A fine-structure genetic and chemical study of the enzyme alkaline
375 phosphatase of E. coli. I. Purification and characterization of alkaline phosphatase. Biochim.
376 Biophys. Acta 38, 470-83.

377 Gee, K.R., Sun, W.C., Bhalgat, M.K., Upson, R.H., Klaubert, D.H., Latham, K A., Haugland, R.P.
378 1999. Fluorogenic substrates based on fluorinated umbelliferones for continuous assays of
379 phosphatases and β-galactosidases. Anal. Biochem. 273, 41-48.

380 German, D.P., Horn, M. H., Gawlicka, A. 2004. Digestive enzyme activities in herbivorous and
381 carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic
382 effects. Physiol. Biochem. Zool. 77, 789–804.

383 Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y., Estévez, A. 2009. Development of digestive
384 enzymes in common dentex *Dentex dentex* during early ontogeny. Aquaculture 287, 381-387.

385 Goldsmith, J.D., Pawel, B., Goldblum, J.R., Pasha, T.L., Roberts, S., Nelson, P., Khurana, J.S., Barr,
386 F.G., Zhang, P.J., 2002. Detection and diagnostic utilization of placental alkaline phosphatase
387 in muscular tissue and tumors with myogenic differentiation. Am. J. Surg. Pathol. 26, 1627-
388 1633.

389 Grotmol, S., Nordvik, K., Kryvi, H., Totland, G.K. 2005. A segmental pattern of alkaline phosphatase
390 activity within the notochord coincides with the initial formation of the vertebral bodies. J. Anat.
391 206, 427-436.

392 Hethey, J. Lai, J., Loutet, S., Martin, M., Tang, V. 2002. Effects of tricine, glycine and tris buffers
393 on alkaline phosphatase activity. J. Exp. Microbiol. Immunol, 2, 33-38.

394 Hidalgo, M.C., Urea, E., Sanz, A., 1999. Comparative study of digestive enzymes in fish with
395 different nutritional habits. Proteolytic and amylase activities. Aquaculture 170, 267–283.

396 Horn, M.H., Gawlicka, A.K., German, D.P., Logothetis, E.A., Cavanagh, J.W., Boyle, K.S., 2006.
397 Structure and function of the stomachless digestive system in three related species of New World
398 silverside fishes (Atherinopsidae) representing herbivory, omnivory, and carnivory. Mar. Biol.
399 149, 1237-1245.

400 Koyama, I., Miura, M., Matsuzaki, H., Sakagishi, Y., Komoda, T. 1987. Sugar-chain heterogeneity
401 of human alkaline phosphatases: differences between normal and tumour-associated isozymes.
402 J. Chromatogr. B Biomed. Sci. Appl. 413, 65-78.

403 Kvåle, A., Mangor-Jensen, A., Moren, M., Espe, M., Hamre, K. 2007. Development and
404 characterisation of some intestinal enzymes in Atlantic cod (*Gadus morhua* L.) and Atlantic
405 halibut (*Hippoglossus hippoglossus* L.) larvae. Aquaculture 264, 457-468.

406 Lallès, J.P. 2010. Intestinal alkaline phosphatase: multiple biological roles in maintenance of
407 intestinal homeostasis and modulation by diet. Nutr. Rev. 68, 323-332.

408 Lazo, J.P., Darias, M.J., Gisbert, E. 2011. Ontogeny of the digestive tract. In: Holt, J.G. (editor),
409 Larval Fish Nutrition, 3-46 pp., Willey-Blackwell, Oxford, UK.

410 Matsuo, Y., Kasahara, Y., Hagiwara, A., Sakakura, Y., Arakawa, T. 2006. Evaluation of larval
411 quality of viviparous scorpionfish *Sebastiscus marmoratus*. Fish. Sci. 72, 948-954.

412 Matusiewicz, M., Dabrowski, K. 1996. Utilization of the bone/liver alkaline phosphatase activity
413 ratio in blood plasma as an indicator of ascorbate deficiency in salmonid fish. Proc. Soc. Exp.
414 Biol. Med. 212, 44-51.

415 McComb, R.B., Bowers Jr, G.N., Posen, S. 1979. Alkaline phosphatase. New York: Plenum Press.

416 Montoya, A., López-Olmeda, J.F., Yúfera, M., Sánchez-Muros, M.J., Sánchez-Vázquez, F.J. 2010.
417 Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead
418 seabream (*Sparus aurata*). Aquaculture 306, 315-321.

419 Moyano F.J, Saenz Rodrigañes, M.A., Diaz,M., Tacon, A.G.J. 2015. Application of *in vitro*
420 digestibility methods in aquaculture: constraints and perspectives, Rev. Aquac. 7, 223–242

421 Parichy, D.M., Elizondo, M.R., Mills, M.G., Gordon, T.N., Engeszer, R.E. 2009. Normal table of
422 postembryonic zebrafish development: staging by externally visible anatomy of the living fish.
423 Dev. Dyn. 238, 2975-3015.

424 Pittman, K., Yúfera, M., Pavlidis, M., Geffen, A.J., Koven, W., Ribeiro, L., Zambonino-Infante, J.L.,
425 Tandler, A. 2013. Fantastically plastic: fish larvae equipped for a new world. Rev. Aquac. 5,
426 S224-S267.

427 Roberts, C.H., Chlebowski, J.F. 1984. Trypsin modification of *Escherichia coli* alkaline phosphatase.
428 J. Biol. Bhem. 259, 729-733.

429 Rong-Qing, Z., Qing-Xi, C., When-Zhu, Z. Jing-Yu, L. Zhong-Lai, Z., Hai-Meng, Z. 2000. Inhibition
430 kinetics of green crab (*Scylla serrata*) alkaline phosphatase activity by dithiothreitol or 2-
431 mercaptoethanol. Int. J. Biochem. Cell Biol. 32, 865-872.

432 Rønnestad, Y., Morais, S., 2008. Digestion. *In*: Finn, R.N. and Kapoor, B.G. (editors), 201-262 pp.,
433 Fish Larval Physiology, Science Publishers, Enfield, USA.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C. 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Rev. Aquac. 5, S59-S98.

Rotllant, G., Moyano, F. J., Andrés, M., Díaz, M., Estévez, A., Gisbert, E. 2008. Evaluation of fluorogenic substrates in the assessment of digestive enzymes in a decapod crustacean *Maja brachydactyla* larvae. Aquaculture 282, 90-96.

Santigosa, E., Sánchez, J., Médale, F., Kaushik, S., Pérez-Sánchez, J., Gallardo, M.A. 2008. Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. Aquaculture 282, 68-74.

Shioi, A., Katagi, M., Okuno, Y., Mori, K., Jono, S., Koyama, H., Nishizawa, Y. 2002. Induction of bone-type alkaline phosphatase in human vascular smooth muscle cells. Circ. Res. 91, 9-16.

Smith, J.L., Madden, L.J., de Jersey, J. 1996. Effect Of Exogenous Cholesterol And Dithiothreitol On The Activity Of Human Liver Microsomal AcylCoenzyme A:Cholesterol Acyltransferase (ACAT). Clin. Chim. Acta 256, 13-25.

Solovyev, M.M., Kashinskaya, E.N., Izvekova, G.I., Gisbert, E., Glupov, V.V. 2014. Feeding habits and ontogenic changes in digestive enzyme patterns in five freshwater teleosts. J. Fish Biol. 85, 1395-412.

Solovyev, M., Gisbert, E. 2016. Influence of time, storage temperature and freeze/thaw cycles on the activity of digestive enzymes from gilthead sea bream (*Sparus aurata*). Fish Physiol. Biochem. 42, 1383-1394.

Solovyev, M.M., Campoverde, C., Öztürk, S., Moreira, C., Diaz, M., Moyano, F.J., Estévez, A., Gisbert, E. 2016. Morphological and functional description of the development of the digestive system in meagre (*Argyrosomus regius*): An integrative approach. Aquaculture 464, 381–391.

458 Solovyev, M.M., Izvekova, G.I., Kashinskaya, E.N., Gisbert, E. 2017. Dependence of pH values in
459 the digestive tract of freshwater fishes on some abiotic and biotic factors. Hydrobiologia *in*
460 *press.*

461 Walter, K., Schutt, C. 1974. Alkaline phosphatase in serum. *In*: Bergmeyer, H.U. (editor), Methods
462 of Enzymatic Analysis 2nd edition, Vol. II, pp 860-864, Academic Press, Inc., NY.

463 Weiss, M. J., Ray, K., Henthorn, P. S., Lamb, B., Kadesch, T., Harris, H., 1988. Structure of the
464 human liver/bone/kidney alkaline phosphatase gene. J. Biol. Chem. 263, 12002-12010.

465 Williams, P.A.M, Barrio, D.A., Etcheverry, S.B., 1999. Interactions of vanadyl (IV) with
466 dithiothreitol and thioglycolic acid. Their interaction on alkaline phosphatase activity. J. Inorg.
467 Biochem. 75, 99-104.

468 Yora, T., Sakagishi, Y. 1986. Comparative biochemical study of alkaline phosphatase isozymes in
469 fish, amphibians, reptiles, birds and mammals. Comp. Biochem. Physiol. 85B, 649-658.

470 Zambonino-Infante, J.L., Cahu, C.L. 2001. Ontogeny of the gastrointestinal tract of marine fish
471 larvae. Comp. Biochem. Physiol. 130C, 477-487.

472 Zambonino-Infante, J.L., Cahu, C.L. 2007. Dietary modulation of some digestive enzymes and
473 metabolic processes in developing marine fish: applications to diet formulation. Aquaculture
474 268, 98-105

475 Zappa, S., Rolland, J.L., Boudrant, J., Flament, D., Gueguen, Y., Dietrich, J. 2011. Characterization
476 of a highly thermostable alkaline phosphatase from the *Euryarchaeon pyrococcus abyssi*. Appl.
477 Env. Microbiol. 2011, 4504–4511.

478 Zouiten, D., Ben Khemis, I., Slaheddin Masmoudi, A., Huelvan, C., Cahu, C. 2011. Comparison of
479 growth, digestive system maturation and skeletal development in sea bass larvae reared in an
480 intensive or a mesocosm system. Aquac. Res. 421, 1723-1736.

481

Figure caption

Figure 1. Recommended protocol for brush border purification from biological samples (modified from Crane et al., 1979). The quantity of tissue for this process (*) depends on the sample (150-200 mg of dissected intestinal tissue; 500-1000 mg of whole specimens). Abbreviations: LAP, leucine-alanine peptidase; TRYP, trypsin; CHYM, chymotrypsin; AMY, amylase; LIP, lipase; BB, brush border; AP, alkaline phosphatase; MAL, maltase; AMN, aminopeptidase-N; γGT, γ-glutamyl transferase.