A meta-analysis for assessing the contributions of trypsin and chymotrypsin as the two major endoproteases in protein hydrolysis in fish intestine

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Abstract: For the majority of fish species, regardless of being gastric or agastric, trypsin and chymotrypsin are known as the two main alkaline proteases responsible for the initial stage of protein hydrolysis in the fish intestine. Although the critical role of these proteases for protein hydrolysis in fish intestine is without doubt, the relative input of each enzyme in protein hydrolysis is still unclear. Data used in the present study has been retrieved from a bibliographic search using the Dimensions application (https://app.dimensions.ai/discover/publication tool). Retrieved articles were carefully inspected to identify whether they contained the description of the development of ontogenetic activities for trypsin, chymotrypsin, and total alkaline proteases in fish intestine. From the list of consulted articles, 21 studies were chosen based on correlation coefficients (Pearson correlation test), and four groups of fish were identified with high significant correlation between 1) the activity of chymotrypsin and total alkaline proteases; 2) the activity of trypsin, chymotrypsin, and total alkaline proteases; 3) the activity of trypsin and total alkaline proteases, and 4) mainly negative correlation between trypsin, chymotrypsin, and total alkaline proteases. These results indicated that the relative inputs of trypsin and chymotrypsin in protein hydrolysis may vary significantly among different fish species, which is a crucial point for proper understanding of species-specific digestive traits in both natural and aquaculture scenarios.

Short title: Trypsin and chymotrypsin input in protein hydrolysis in fish intestine

Keywords: total alkaline proteases; trypsin; chymotrypsin; feed formulation; intestine; fish digestive physiology

1. Introduction

Digestion is a multi-level complex process that consists of the physico-chemical degradation and absorption of a wide number of organic and inorganic substances ingested by the organism. The key role in the degradation of food items is related to digestive enzymes, which are characterized by their origin (i.e., pancreas, stomach, intestine, food items, symbiotic microbiota among others), substrate specificity (proteins, carbohydrates, lipids, etc), and gut localization (lumen, brush border, intracellular), dependence on pH, cofactors required for activation, etc. As a result, the spectrum of digestive enzymes that could be found in fish gut is very diverse and enables hydrolysis of a wide variety of substrates from food items, facilitating nutrient absorption by the organism. The digestive system of vertebrates is adapted through evolution to maximize nutrient uptake and energy from each available food substrate. Proteins, one of the main substrates obtained by fish from the diet, vary in terms of their molecular weight, size, amino acid composition, solubility, surface hydrophobicity and chemical modifications (i.e., phosphorylation, glycosylation, etc.) among others. For the majority of fish species, trypsin and chymotrypsin are known as the two main alkaline digestive proteases responsible for the initial stage of protein hydrolysis in the intestine of both gastric and agastric fish species. Both proteases are synthesized in the exocrine pancreas and accumulated in non-active zymogen forms (trypsinogen and chymotrypsinogen) and, then they are discharged in the intestinal lumen where enterokinase cleaves a short peptide from trypsinogen converting it to an active form (trypsin). Furthermore, the trypsin autoactivates/activates other molecules of trypsinogen (Kay and Kassell, 1971), chymotrypsinogens (Appel, 1986), procarboxypeptidases A and B (Keller et al., 1958), and several other hydrolases (Williams, 2004). Trypsin and chymotrypsin are characterized by several substrate specificities, cleaving different peptide bonds in proteins and polypeptides; for instance, trypsin predominantly cleaves proteins at the carboxyl side of amino acids like lysine and arginine, except when either is bound to a C-terminal proline, whereas chymotrypsin preferentially cleaves peptide amide bonds at the carboxyl side of aromatic amino acids like tyrosine, tryptophan, and phenylalanine (Heu et al., 1995). Moreover, for different proteases, the most relevant biochemical characteristics affecting protein hydrolysis are the number of cleavage sites and secondary enzyme specificity (i.e., enzyme preferences resulting from neighboring amino acids) that may change from different proteins (Deng et al., 2019). Both of these proteases are found in several isoforms/isoenzymes in fish gut (Heu et al., 1995; Cohen et al., 1981; Chong et al., 2002; Moutou et al., 2004; Rungruangsak-Torrissen et al., 2006). These isoforms/isoenzymes are characterized by different kinetic parameters, optimal pH values and temperature, and other variables and show different stabilities under several physicochemical parameters of the chyme (i.e., pH, temperature, ion concentrations, osmolarity, bile acid composition and concentration, etc.). The synthesis and production of different isoforms/isoenzymes are considered as one of adaptative mechanisms of fish to enhance their digestive capacities under various biotic and abiotic factors such as water temperature, pH, food supply, water salinity, among others (Zhou and Budge, 2011).

Both trypsin and chymotrypsin are normally detected in the digestive system of fish at their early life stages (Vega-Orellana et al., 2006; Jimenez-Martinez et al., 2012; Solovyev et al., 2016; Mente et al., 2017). For several fish species, the activity of both proteolytic enzymes may be detected at hatching before the exocrine pancreas has fully completed its morphogenesis (Jimenez-Martinez et al., 2012; Alvarez-González et al., 2008; Mello et al., 2021). Furthermore, both proteases may demonstrate several peaks of activity during fish ontogeny, which are generally correlated to the morphogenesis of the digestive organs (i.e., pancreas, stomach) and/or shifts in the diet. As the ontogeny of digestive enzymes is genetically preprogrammed (Zambonino Infante and Cahu, 2001), their observed peaks of activity at different stages of development are considered to be related to various substrate demands (i.e., proteins, carbohydrates, lipids) based on the capacities of the fish digestive system. In the same time, the above-mentioned changes in proteolytic activity may also be modulated by the inclusion of dietary proteins (Pérez-Jiménez et al., 2009). The effect of dietary proteins on the pancreatic proteolytic enzymes depends on the proteins concentrations and sources (Rodiles et al., 2012; Mente et al., 2017; Abbasi et al., 2020; Fronte et al., 2021), the stage of fish ontogeny (Canada et al., 2017), feeding protocol (Solovyev and Gisbert, 2021), among others.. However, the relationship between dietary crude protein and alkaline protease activities is not always linear. For instance, the level of activity of both trypsin and chymotrypsin in tilapia juveniles (*Oreochromis* sp.) increased when the concentration of crude protein changed from 24 to 35%, whereas the activity of the above-mentioned enzymes decreased when diets contained 42% crude protein (Santos et al., 2020). Similar results were obtained for *Culter mongolicus* fingerlings when the activity of pancreatic proteases was positively modulated by dietary protein inclusion until certain level (Qian et al., 2022).

Despite there being no doubt about the critical role of these proteases for protein hydrolysis in fish intestine, the relative input of each enzyme in protein hydrolysis is still unclear and disputable (Moutou et al., 2004; Alvarez-González et al., 2008; Lazo et al., 2007; López-Ramírez et al., 2011). Several different approaches have been applied in order to estimate the relative importance of trypsin and chymotrypsin in protein degradation. In some studies, the direct comparison of activity values between both proteases has been conducted (Moutou et al., 2004; Olatunde and Ogunbiyi, 1977; Jónás et al., 1983; Uscanga et al., 2010). It is well known that under optimal conditions (i.e., pH, temperature, concentration of enzyme activators, substrate concentration, among others), the activity of any digestive enzyme depends on its concentration and turnover number (Bisswanger, 2014). Unfortunately, there is no information about turnover numbers for trypsin and chymotrypsin from the majority of fish species. Moreover, Lazo et al. (2007) mentioned that the direct estimation of the relative contribution of trypsin and chymotrypsin to protein digestion is not possible since different specific substrates are applied in their biochemical spectrophotometric quantification. At the same time, when the activity of any digestive enzyme, for example trypsin or chymotrypsin, is estimated for different fish species, the reaction buffer used for assessing enzyme activity is generally formulated with a "standard" buffer with a fixed level of pH, concentration of ions such as Ca^{2+} ($CaCl_2$) and Na^+ (NaCl), total osmolarity, and some other parameters that may not have been optimized for the target species and the enzyme of interest. The development and use of "universal" protocols for the quantification of trypsin, chymotrypsin or any other digestive enzymes has the limitation that it does not take into account that each enzyme has species-specific functional properties. For instance, trypsins obtained from different fish species may have different optimal pH values, they may be inhibited and/or activated by different concentrations of Na^+ and Ca^{2+} ions, and/or osmolarity levels (Dos Santos et al., 2016; Silva et al., 2011; Liu et al., 2012; Shi et al., 2007; Khangembam et al., 2012). All these factors will affect the activity of the enzyme in different ways by means of changing their activities in unpredictable manner, which make straight-forward comparisons unclear. As a result, activity levels will be obtained with biases that, consequently, may potentially lead to wrong conclusions. In this sense, Yúfera et al. (2018) directly compared the specific activity of trypsin among 15 fish species obtained by different studies and showed that the specific trypsin activity ranged over more than 50-fold among fish species and therefore concluded that direct comparisons of absolute values among species should be very restricted. In order to overcome such limitations, it should be recommended that the functional properties of any targeted enzyme be determined in advance. Unfortunately, such time and resource consuming preliminary studies are ignored in many cases and the activity of enzymes is measured using "standard" protocols; whereas the most accurate way for performing these analyses would be to create, based on a known scheme, species-specific protocols for key digestive enzymes based on their biochemical features. Although conducting this approach may be impossible considering the large diversity in fish species, it seems reasonable that it should be conducted at least for economically valuable species due to the impact that nutrition has on fish growth and performance under farming conditions, or developing alternative approaches to bypass such preliminary time-consuming studies.

Among the different approaches that may be used for properly estimating the relative importance of different enzymes on the digestive processes, the use of specific inhibitors is a conventional and useful procedure. For instance, applying the specific inhibitors for each protease in the hydrolysis of model protein may help to proper understanding their relative input on protein digestion. In this context, several specific synthetic inhibitors, e.g. TLCK (Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride) and TPCK (N-p-Tosyl-L-phenylalanine chloromethyl ketone) / ZPCK (N-Carbobenzoxy-L-phenylalanyl-chloromethyl ketone) / CHYM (chymostatin), are normally used in order to inhibit the activity of trypsin and chymotrypsin, respectively (Lazo et al., 2007; Martinez and Serra, 1989; Alarcón et al., 1998; Essed et al., 2002; García-Carreño et al., 2002; Natalia et al., 2004; Sáenz de Rodrigáñez et al., 2005).

Although these inhibitors will decrease the activity of these targeted enzymes, the level of such inhibition could be species-specific (Eshel et al., 1993), and its efficiency will depend on several factors such as the enzyme:inhibitor ratio, mutations or deletions in specific binding site of the enzyme, and/or the fish species considered (Martinez and Serra, 1989, García-Carreño et al., 2002, Natalia et al., 2004, Zhou et al., 1989; Turk et al., 2002). As a result, in many cases the sum of inhibition activities of trypsin and chymotrypsin together is more that 100%. For example, the sum of the percentage of inhibition for trypsin and chymotrypsin by TLCK and TPCK was 129.2% (Natalia et al., 2004), which complicates the proper interpretation of the results with regard to the relative importance of each alkaline protease in the digestive process.

Another possible approach to determine the relative importance of these endoproteases is to compare the level of gene expression for each one. It is generally accepted that the higher the gene expression level, the higher is the expected enzyme activity. However, the abundance of gene transcripts is not always correlated to the amount of protein transcribed, since mRNA levels may be post-transcriptionally and/or translationally regulated, or there may even exist protein degradation/turnover. In this sense, the cellular concentrations of proteins correlate with the abundances of their corresponding mRNAs, but not strongly. Some authors have shown a squared Pearson correlation coefficient of ca. 0.40 between protein and mRNA levels, which implies that ~40% of the variation in protein concentration, can be explained by knowing mRNA abundances (Vogel and Marcotte, 2012).

Despite the fact that there are various methods for determining the activity of trypsin and chymotrypsin for many economically valuable fish species, and the specific biochemical features of these enzymes are known, the appropriate approach showing the relative inputs of trypsin and chymotrypsin in protein digestion is still needed. This information would help to better understand the digestive capacity of a given species and meet their specific nutritional protein demands that vary during morphological and physiological changes in the digestive system of fish during their ontogeny. This may be of special importance during early life stages of development when acid digestion may not exist or only partially achieved. Thus, understanding the relative inputs of trypsin and chymotrypsin in protein digestion in fish larvae coupled with information associated with fish digestive physiology may be of interest for proper formulation of compound diets in a stage- and species-specific way, since each endoprotease has different cleavage sites.

In the present study, for proper understanding of the above-mentioned methodological shortcomings, we have estimated the relative importance of trypsin and chymotrypsin in protein digestion for different fish species based on the correlation analysis among activity levels of trypsin, chymotrypsin, and total alkaline proteases from available literature. In order to achieve this aim, we have put forward two hypotheses: 1) the development of activity of total alkaline proteases during fish ontogeny will be substantially dependent on the activity of trypsin and chymotrypsin as these are the major alkaline proteases when compared to metalloproteases and cysteine proteases that are also detected in fish intestine by inhibitor analyses; and 2) as the role of trypsin or chymotrypsin in protein hydrolysis increases, then there will be a higher level of similarities between trypsin/chymotrypsin and total alkaline proteases activities during fish ontogeny as expressed by means of correlation coefficients.

2. Materials and methods

Data used in the present study has been retrieved from a bibliographic search using the Dimensions application (https://app.dimensions.ai/discover/publication tool). The following key words in different combinations were used for this bibliographic search: “trypsin”, “chymotrypsin”, “alkaline proteases”, “fish”, “larva”, “ontogeny”, and “development”. Retrieved articles were carefully inspected to identify whether they contained the description of the development of ontogenetic activities for trypsin, chymotrypsin, and alkaline proteases. Among the list of consulted articles (85), authors have chosen a total of 21 studies in order to run correlation analysis that included 19 fish species and 2 fish hybrids (raw data may be find in the Supplementary file 1). The rest of the articles were excluded from the analysis because of the dataset for one of proteases (trypsin or chymotrypsin or total alkaline protease activities) was absent. In order to support the obtained results by the correlation analysis, we have chosen 13 articles where the inputs for trypsin and chymotrypsin in protein digestion were available by means of using specific protease inhibitors using casein as a protein substrate. Unfortunately, the low number of available data did not allow us to estimate the effect of casein (azo-casein) concentrations (ranged between 0.5-8.0%) on the percentages of inhibition. Thus, we have only shown the concentration of model protein in Table as a supporting information. In addition, we have also provided information about preferable water salinity, rearing, and feeding conditions as well as feeding habits in order to characterize each studied fish species and assumed that these conditions are optimal for studied fish development.

When data were not presented in numerical values within tables, activity values for trypsin, chymotrypsin and total alkaline proteases were extracted from graphs to the closest unit from each selected paper of interest. The correlation analysis among activity for trypsin, chymotrypsin, and total alkaline proteases was conducted by means of the Pearson correlation test and a level of significance of $p < 0.10$. Principal Components Analysis (PCA) was calculated based on Pearson correlation coefficients (*r* values) between trypsin and total alkaline proteases, chymotrypsin and total alkaline proteases, as well as trypsin and chymotrypsin. All calculations were done using PAST v. 3.16 (Hammer et al., 2001) and Microsoft Office Excel.

3. Results

3.1. Correlation analysis between enzyme activities

All Pearson correlation coefficients (*r*) calculated among values for the activities of trypsin, chymotrypsin, and total alkaline proteases during ontogeny of different fish species are given in Figure 1 and Supplementary file Table A.

Figure 1. Heat map based on *r* values calculated with Pearson correlation analysis among the activity of trypsin (Tryp), chymotrypsin (Chymo), and total alkaline proteases (TAP) during ontogenetic development of different fish species. Correlation coefficients that were statistically significant ($p < 0.1$) are marked by white asterisks.

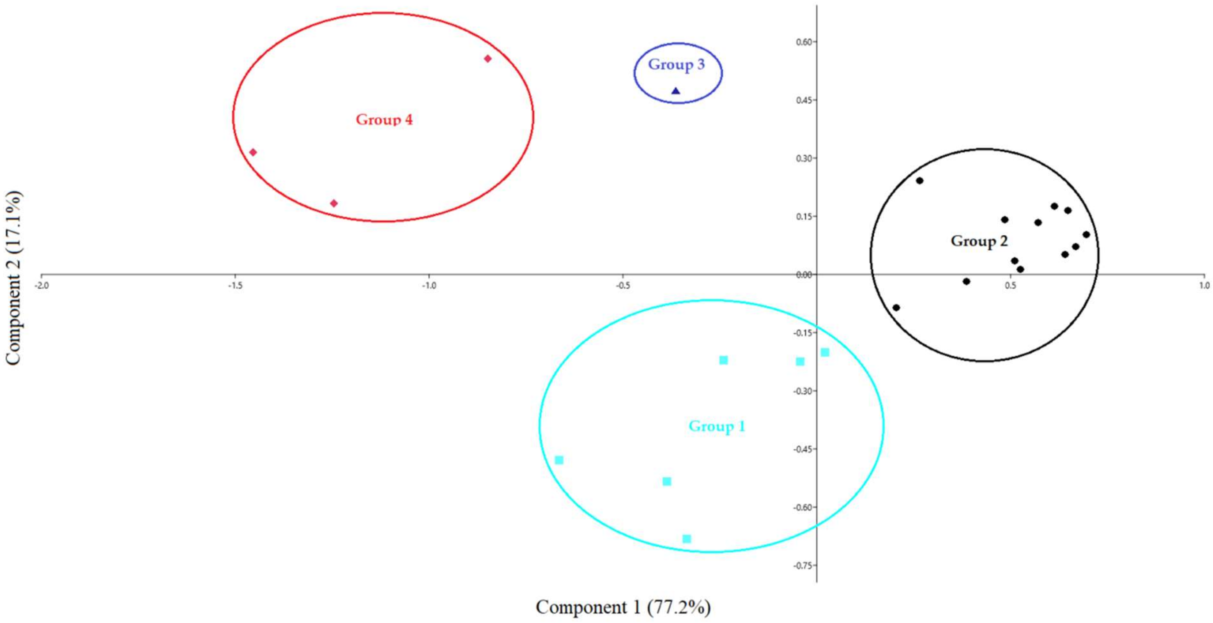
Group	Tryp/TAP	Chymo/TAP	Tryp/Chymo	Species (Family)	References
Group 1	-0,17	0,61 *	0,02	*Centropomus undecimalis* (Centropomidae)	(Jimenez-Martinez et al. 2012)
	0,33	0,64 ***	0,16	*C. viridis* (Centropomidae)	(Hernández-López et al. 2021)
	0,37	0,80 ***	0,50	*Argyrosomus regius* (Sciaenidae)	(Solovyev et al. 2016)
	-0,05	0,97 ****	0,10	*Archocentrus nigrofasciatus* (Cichlidae)	(Mente et al. 2017)
	-0,08	0,83 ****	0,23	*Petenia splendida* (Cichlidae)	(Uscanga-Martínez et al. 2011)
	0,26	0,79 ***	0,55 *	*Paralichthys californicus* (Paralichthyidae)	(Alvarez-González et al. 2006)
Group 2	0,59 ***	0,81 ****	0,56 ***	*Ocyurus chrysurus* (Lutjanidae)	(Ahumada-Hernández et al. 2014)
	0,74 **	0,91 ****	0,91 ****	*Atractosteus tropicus* (Lepisosteidae)	(Frías-Quintana et al. 2015)
	0,93 ***	0,96 ***	0,83 **	*Catla catla* (Cyprinidae)	(Rathore et al. 2005)
	0,71 **	0,85 ***	0,72 **	*C. catla* (Cyprinidae)	(Khangembam et al. 2012)
	0,86 ****	0,92 ***	0,73 ***	*Cichlasoma dimerus* (Cichlidae)	(Toledo-Solís et al. 2021)
	0,88 **	0,59 ***	0,53 **	*C. trimaculatum* (Cichlidae)	(Toledo-Solís et al. 2015)
	0,92 ****	0,96 ****	0,98 ****	*Cirrhinus mrigala* (Cyprinidae)	(Chakrabarti, Rathore 2010)
	0,90 ***	0,97 ****	0,94 ***	*Odontesthes bonariensis* (Atherinopsidae)	(Pérez Sirkin et al. 2020)
	0,93 ****	0,85 ****	0,82 ****	*Hypophthalmichthys molitrix* × *H. nobilis* (Cyprinidae)	(Chakrabarti et al. 2006b)
	0,92 ****	0,85 ****	0,94 ****	*Labeo rohita* (Cyprinidae)	(Chakrabarti, Rathore 2006a)
	0,97 ****	0,78 ***	0,64 **	*Paralabrax maculatofasciatus* (Serranidae)	(Alvarez-González et al. 2008)
	0,94 **	0,88 **	0,95 **	*Solea solea* (Soleidae)	(Clark et al. 1986)
Group 3	0,65 ***	-0,01	0,24	*C. urophthalmus* (Cichlidae)	(López-Ramírez et al. 2011)
Group 4	-0,57	-0,22	0,48	*Pseudoplatystoma punctifer* (Pimelodidae)	(Castro-Ruiz et al. 2019)
	-0,38	-0,49 **	0,03	*P. reticulatum* (Pimelodidae)	(Mello et al. 2021)
	0,10	-0,32	0,45 *	*P. corruscans x P. reticulatum* (Pimelodidae)	(Mello et al. 2021)

Value: 1.00, 0.25, 0.00, -0.25, -1.00

Tryp – trypsin, Chymo – chymotrypsin, TAP – total alkaline proteases. The asterisks denote ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$, *$p < 0.10$.

Based on the similarity and correlationship (-1 < r > 1) of the correlation coefficients and Principal Components Analysis (PCA), four groups of fish were identified (Figure 2). These groups are described as follow: 1) high positive significant correlationship (r = 0.61 – 0.97) between the activity of chymotrypsin and total alkaline proteases (6 species – 28.6%); 2) high positive significant correlationship between the activity of trypsin (r = 0.59 – 0.97), chymotrypsin (r = 0.78 – 0.97), and total alkaline proteases (10 species and 1 hybrid species – 52.4%); 3) high positive significant correlationship (r = 0.65) between the activity of trypsin and total alkaline proteases (1 species – 4.8%); and 4) negative correlation between trypsin (r = -0.38 – -0.57), chymotrypsin (r = -0.22 – -0.49), and total alkaline proteases (2 species and 1 hybrid species – 14.3%).

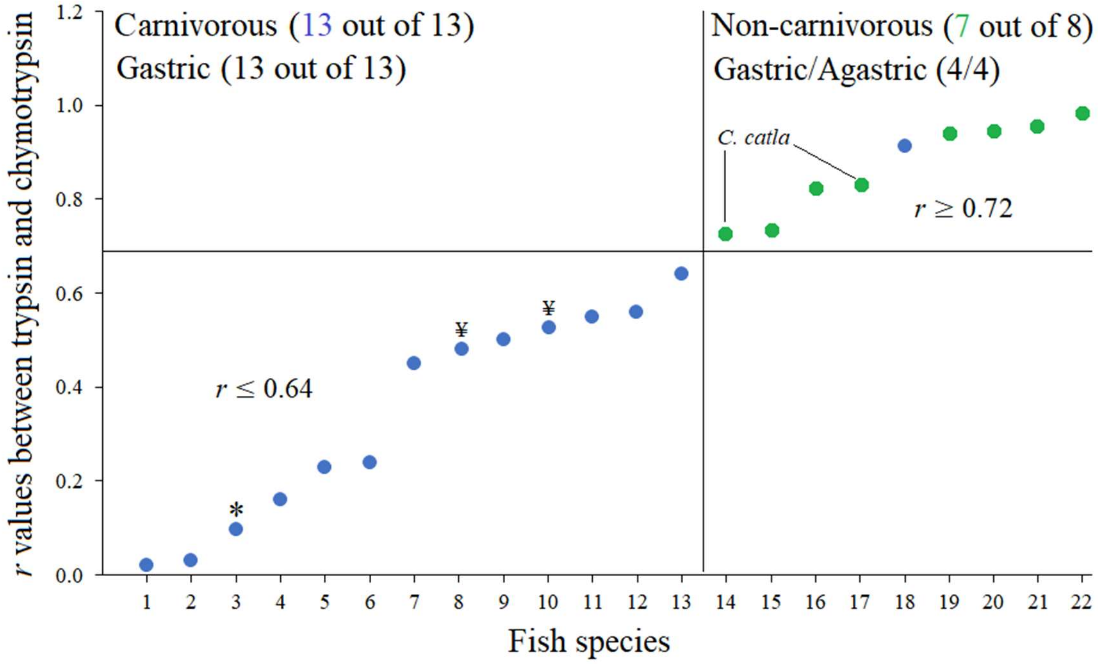
Figure 2. Principal Components Analysis (PCA) based on correlation coefficients between trypsin and total alkaline proteases, chymotrypsin and total alkaline proteases, trypsin and chymotrypsin.



It is of special relevance that all six species with high inputs of only chymotrypsin (group 1) and trypsin activities (group 3) regarding comparison to total alkaline proteases belonged to gastric fish species with predominated carnivorous feeding habits. However, all fish species from group 2 for which the effect of both enzymes (trypsin and chymotrypsin) on the activity of total alkaline proteases was significant and similar, belonged to both gastric and agastric species, which were characterized by different feeding habits (carnivorous, zooplanktivorous, benthivorous, herbivorous, and omnivorous). The group 4 characterized by negative correlationship among studied proteases only included Amazonian carnivorous-omnivorous catfishes (Supplementary file 2 - Table A).

The correlationship between trypsin and chymotrypsin activities was positive for all studied fishes. In 38.1% of the cases (7 species and 1 hybrid), the correlationship was irrelevant and low or moderate (r = 0.02–0.48) whereas in 61.9% of the cases (12 species and 1 hybrid), the correlationship between the activity of both proteases was high (r = 0.50–0.98). It has to be noted that the r values between trypsin and chymotrypsin were lower for gastric carnivorous fishes (r = 0.02–0.64, mean r = 0.35) with one exception, the tropical gar (*A. tropicus*) (r = 0.91), while Pearson correlation values for non-carnivorous fishes (6 species and 1 hybrids) were higher (r = 0.73–0.98, mean r = 0.88) (Figure 3).

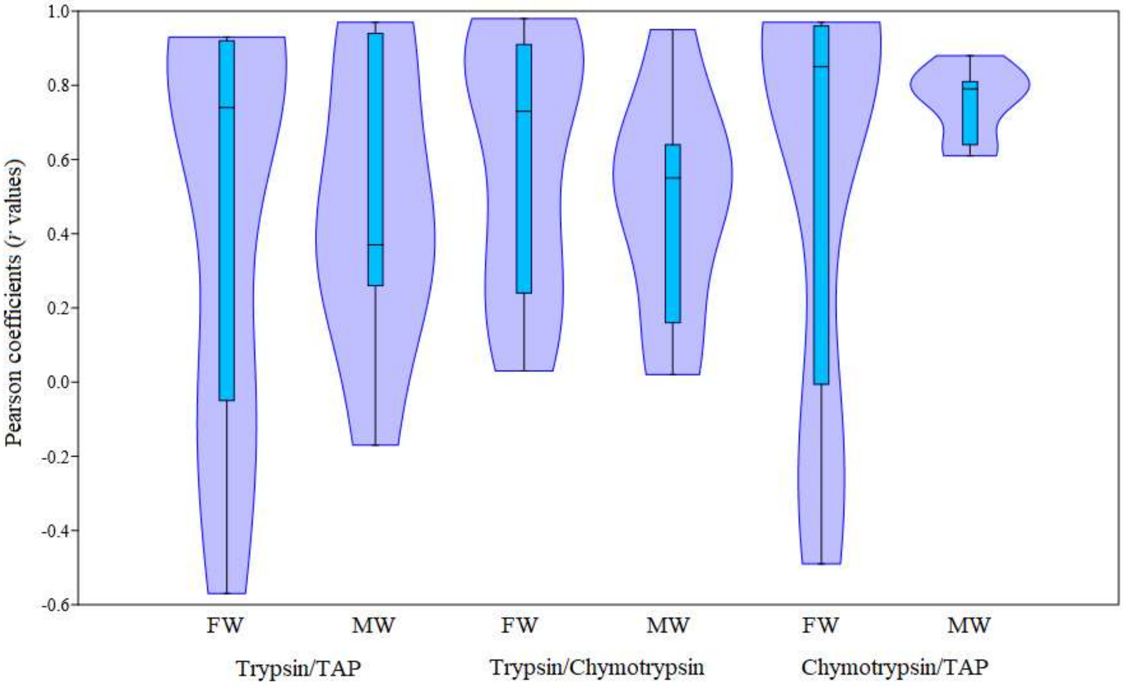
Figure 3. Pearson correlationship (r values) between trypsin and chymotrypsin for studied fish species.



1. *Centropomus undecimalis*, 2. *Pseudoplatystoma reticulatum*, 3. *Archocentrus nigrofasciatus*, 4. *C. viridis*, 5. *Petenia splendida*, 6. *Cichlasoma urophthalmus*, 7. *P. corruscans* × *P. reticulatum*, 8. *P. punctifer*, 9. *Argyrosomus regius*, 10. *C. trimaculatum*, 11. *Paralichthys californicus*, 12. *Ocyurus chrysurus*, 13. *Paralabrax maculatofasciatus*, 14. *Catla catla* (Khangembam et al., 2017), 15. *C. dimerus*, 16. *Hypophthalmichthys molitrix* × *H. nobilis*, 17. *C. catla* (Rathore et al., 2005), 18. *Atractosteus tropicus*, 19. *Odontesthes bonariensis*, 20. *Labeo rohita*, 21. *Solea solea*, 22. *Cirrhinus mrigala*; Blue circle – carnivorous species, green circle – non-carnivorous species. * – carnivorous-benthivorous, ¥ – carnivorous-omnivorous.

The correlationship between chymotrypsin and total alkaline proteases activities was positive for all studied marine fishes with *r* values that ranged from 0.61 to 0.88. In the same time, for freshwater fishes the *r* values between chymotrypsin and total alkaline proteases activities were widely ranged (*r* = -0.49 – 0.97). Correlationships between trypsin and total alkaline proteases as well as trypsin and chymotrypsin activities were similar between studied marine and freshwater fishes (Figure 4).

Figure 4. Pearson correlationship (*r* values) between chymotrypsin/total alkaline proteases (TAP), trypsin/chymotrypsin, and trypsin/total alkaline proteases for studied marine and freshwater fishes. Abbreviations: MW: marine fish species, FW, freshwater fish species.



3.2. Specific inhibitor analysis

The relative input of trypsin and chymotrypsin in the hydrolysis of proteins as estimated by specific inhibitors like TLCK and TPCK/ZPCK/CHYM, respectively is presented in Table X. Based on similarity of percentages of inhibition activity, three fish groups were identified as follows: 1) high percentage of trypsin inhibition (*B. orbignyanus* and *C. viridis* – 13.3%); 2) high percentage of chymotrypsin inhibition (*D. dentex*, *T. thynnus*, and *M. chrysops* × *saxatilis* – 20%); 3) similar percentage of trypsin and chymotrypsin inhibition (all other fishes not included in groups 1 and 2 – 66.7%).

Table X. The effect of the specific synthetic inhibitors on trypsin and chymotrypsin activities in the gut of different fish species. In all cases, TLCK and TPCK were used as the specific inhibitors for trypsin and chymotrypsin activity, respectively, when additionally, other inhibitors (ZPCK and CHYM) for chymotrypsin were used, they were indicated in parenthesis. The data are expressed as mean ± SE.

Species (Family)	Percent of inhibited activity		Model protein	References
	Trypsin	Chymotrypsin		
Common dentex *Dentex dentex* (Linnaeus, 1758) (Sparidae)	6.0 ± 4.7%	26.2 ± 8.9% (TPCK) 40.5 ± 7.5% (CHYM) 36.1 ± 7.5% (ZPCK)	0.5% casein	(Alarcón et al., 1998)
Gilthead seabream *Sparus aurata* (Linnaeus, 1758) (Sparidae)	16.8 ± 2.7%	19.5 ± 7.3% (TPCK) 45.4 ± 6.7% (CHYM) 26.2 ± 5.4% (ZPCK)	0.5% casein	(Alarcón et al., 1998)
Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus, 1758) (Scombridae)	7.0%	29.0% (TPCK) 32.0% (ZPCK)	0.5% casein	(Essed et al., 2002)
Red drum *Sciaenops ocellatus* (Linnaeus, 1766) (Sciaenidae)	26%	30% (TPCK)	2.0% azo-casein	(Lazo et al., 2007)
Hypophthalmichthys molitrix × *nobilis* (Cyprinidae)	45.1-55.5%	35.8-48.2% (TPCK)	1.0% azo-casein	(Chakrabarti et al., 2006b)
Labeo rohita (Cyprinidae)	41.1–52.4%	28.0–44.5% (TPCK)	1.0% azo-casein	(Chakrabarti et al., 2006a)
European anchovy	96.0%	98.0%	8.0% casein	(Martinez and Serra, 1989)

Engraulis encrasicolus (Linnaeus, 1758) (Engraulidae)				
Atractosteus tropicus (Lepisosteidae)	7.2%	9.4%	1.0% casein	(Guerrero-Zárate et al., 2014)
Blue discus *Symphysodon aequifasciatus* (Pellegrin, 1904) (Cichlidae)	46.4 ± 5.3%	39.7 ± 6.8%	1.0% casein	(Chong et al., 2002)
Senegalese sole *Solea senegalensis* (Kaup, 1858) (Soleidae)	35.6-41.5%	4.3-6.0% (TPCK) 28.7-29.4% (ZPCK)	0.5% casein	(Sáenz de Rodrigáñez et al., 2005)
Asian bony tongue *Scleropages formosus* (Müller & Schlegel, 1840) (Osteoglossidae)	71.5 ± 3.5%	57.7 ± 2.8%	1.0% azo-casein	(Natalia et al., 2004)
Brycon orbignyanus (Valenciennes, 1850) (Bryconidae)	52.0 ± 2.0%	3.0 ± 0.2%	1.5% azo-casein	(García-Carreño et al., 2002)
Centropomus viridis (Centropomidae)	69.8%	9.0%	1.0% casein	(Hernández-López et al., 2021)
European seabass *Dicentrarchus labrax* (Linnaeus, 1758) (Moronidae)	≈ 38%	≈ 48%	0.95% casein	(Eshel et al., 1993)
White bass x striped bass *Morone chrysops* (Rafinesque, 1820) x *M. saxatilis* (Walbaum, 1792) (Moronidae)	≈ 20%	≈ 37%	0.95% casein	(Eshel et al., 1993)

4. Discussion

The relative importance of trypsin and chymotrypsin in alkaline protein digestion may change during fish ontogeny (López-Ramírez et al., 2011), whereas it also depends on rearing and feeding conditions (Rungruangsak-Torrissen et al., 2006). Jónás et al. (1983) have shown that the activity of trypsin was about four times higher in comparison to the activity of chymotrypsin in the intestine of sheatfish (*Silurus glanis* Linnaeus, 1758), whereas in Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) the activity of chymotrypsin was two times higher than the activity of trypsin. As it was mentioned above, the estimation of inputs of both trypsin and chymotrypsin in protein digestion using the direct comparison of their activity levels between each other is unreliable. This approach requires information about turnover numbers based on analysis of the purified enzymes as well as the specific optimal activity conditions (pH, ion concentrations, osmolarity, etc.). Unfortunately, this information is only available in a very reduced number of species (Heu et al., 1995; Jónás et al., 1983; Hinsui et al., 2006). Moreover, the quality of enzyme purification depends on the applied protocol, and consequently, it also affects the turnover number (Hinsui et al., 2006; Barkia et al., 2010; Stefansson et al., 2010). But even if all these required biochemical characteristics were determined, the different enzyme specific substrates applied would not allow for a direct comparison between both endoproteases (Lazo et al., 2007).

The use of enzyme inhibitors is another approach for the characterization of contribution of trypsin and chymotrypsin activity to protein digestion (Heu et al., 1995; Alarcón et al., 1998). As different inhibitors have different inhibitory mechanisms and there may exist several inhibition constants for the same enzyme (Ferguson et al., 2022), the degree of enzyme inhibition activity may also change in a significant way (Chong et al., 2002; Guerrero-Zárate et al., 2014). This fact has significantly restricted the determination of the input of different enzymes in the general digestive process. However, based on the analysis of the inhibitory effects of specific inhibitors in trypsin and chymotrypsin in fish gut from the literature, relatively high inputs were noted for both proteases as described in Table. On one hand, the significant role of trypsin in protein hydrolysis in fish intestine is not surprising, since trypsin may digest a number of different proteins in fish diets and also activates other pancreatic proteases. On other hand, the inhibition efficiency depends largely on different digestive variables, as well as on the inhibitor considered. In the present study, we have not used data based on the use of soybean trypsin inhibitor (SBTI), because it has been shown that this inhibitor affects the activity of both trypsin and chymotrypsin (Martinez and Serra, 1989). Except for two fish species belonging to the group 1 (*B. orbignyanus* and *C. viridis*; Table? Figure 1), we did not find that the input of trypsin activity was significant when considered alone. At the same time, the input of chymotrypsin activity alone in casein digestion was found to be significant only for *D. dentex*, *T. thynnus*, and *M. chrysops* × *M. saxatilis* (group 2; Table). For the majority of considered fish species, both proteases showed a significant contribution in protein digestion (group 3; Table). This result is in agreement with our correlation analysis that also showed that the majority of fish species had significant inputs for both trypsin and chymotrypsin activities in protein digestion (Figure 1 and 2; Supplementary file raw data). One of the main limitations of the application of the inhibitory analysis as a tool for determination of inputs of protease activities in protein digestion is the different specificity of inhibitors to target enzymes. For instance, the percentage of inhibition in chymotrypsin activity was significantly different when TPCK (4.3-6.0%) or ZPCK (28.7-29.4%) inhibitors were used (Sáenz de Rodrigáñez et al., 2005). Thus, depending on the inhibitor considered, the reader may get misleading conclusions depending on the study consulted. In this sense, if only TPCK was used as a specific chymotrypsin inhibitor, the reader may conclude that the input of chymotrypsin in casein digestion is no more than 6.0% for *S. senegalensis*, whereas when another specific inhibitor (ZPCK) was used for such analyses, chymotrypsin contribution ranged from 28.7 to 29.4%, values that were similar to those observed for trypsin (35.6–41.5%) (Sáenz de Rodrigáñez et al., 2005).

According to our correlation analysis, we demonstrated that the inputs of both trypsin and chymotrypsin in the activity of total alkaline proteases had a similar importance in terms of protein digestion as *r* values indicated for half of the studied fish species (10 out 19 species and 2 hybrids, group 2 in Figure 1 and 2 and Supplementary file Table A). In addition, we have also found a good agreement in data obtained from different studies (Rathore et al., 2005 and Khangembam et al., 2017), but for the same fish species (*C. catla*) that confirmed the reproducibility of our obtained results. High inputs of both trypsin and chymotrypsin in the activity of total alkaline proteases is also consistent with data from inhibitor analyses that showed the similar and high percentages of inhibition for both proteases for the following species: *E. encrasicholus* (Martinez and Serra, 1989), *S. aequifasciata* (Chong et al., 2002), *H. molitrix* × *H. nobilis* (Chakrabarti et al., 2006b), and *S. ocellatus* (Lazo et al., 2007). Unexpectedly, only for *C. urophthalmus* was there shown a high positive significant correlationship between activity of total alkaline proteases and trypsin ($r = 0.65$; $p < 0.01$) and slightly negative, but not significant, correlationship between the activity of chymotrypsin and total alkaline proteases ($r = -0.006$ at $p = 0.98$) (group 3; Figure 1 and 2 and Supplementary file Table A). Moreover, six fish species (28.6%) showed a significant contribution only for chymotrypsin activity in protein digestion (group 1; Figure 1 and 2 and Supplementary file Table A). This observation was also partially supported by data from enzyme inhibitor analyses, since for several species like *T. thynnus* (Essed et al., 2002), *D. dentex*, and *S. aurata* (Alarcón et al., 1998) the percentage of chymotrypsin activity inhibited was higher when compared to that of trypsin. We may assume that the significant prevalence of trypsin or chymotrypsin alone in protein hydrolysis in fish intestine is less common among fishes when compared to fish species for which both proteases have relatively high activity levels. Unfortunately, we could not extend these analyses, since there were only three fish species (*C. viridis*, *A. tropicus,* and *L. rohita*), one hybrid (*H. molitrix* × *H. nobilis*), and one genus (*Solea*) for which the data of correlation and inhibitory analyses were available, which highlights the importance of conducting species-specific studies on the proper characterization of digestive enzymes. In case of *C. viridis*, chymotrypsin showed a significant contribution in the activity of total alkaline proteases between 1 and 40 DAH ($r = 0.64$, $p = 0.003$; Supplementary file table A), whereas on the contrary, the results based on the inhibitory analysis for the same species demonstrated a higher input of trypsin than chymotrypsin activity at 55 DAH (69.8% by 9.0%, respectively; Table) (Hernández-López et al., 2021). However, it is important to mention that data from this study may not be directly comparable, since the correlation analysis was computed with the integrated results based on ontogeny data (1-40 DAH), whereas data from the inhibitor analysis was taken only from one age point at the juvenile stage (55 DAH). It also needs to be mentioned that at 55 DAH, the relative importance of studied proteases could be changed due to physiological alterations or changes in diets (fish were only fed by a compound dry diet after 35 DAH). For instance, the percentage of inhibited trypsin and chymotrypsin activities changed during ontogeny in the hybrid *H. molitrix* × *H. nobilis* (Chakrabarti et al., 2006b) and *S. ocellatus* (Applebaum et al., 2001). Moreover, using only one specific inhibitor for chymotrypsin may lead to underestimation of chymotrypsin input in protein digestion for this species as it has been shown for *S. aurata* (Alarcón et al., 1998) and *S. senegalensis* (Sáenz de Rodrigáñez et al., 2005). For the other four cases (*H. molitrix* × *H. nobilis, A. tropicus*, *L. rohita*, and *Solea* spp.), the results obtained by correlation and inhibitory analyses were in agreement. Such good concordance in results obtained by two different approaches demonstrated that inhibitory analysis is a suitable approach when specific inhibitors are correctly targeted towards selected enzymes. But this assumption needs to be supported by additional approaches, because in the case of *D. dentex* we were not able to establish whether the real trypsin input was only of 6%, or because of the inhibitor in use being unable to inhibit trypsin. Unexpectedly, for three Amazonian catfishes we have found a negative correlationships between total alkaline proteases and both trypsin and chymotrypsin (group 4; Supplementary file Table A). It means that the total alkaline protease activity was mainly due to cysteine- or/and metallo-proteases. The cystene-proteases are believed to have low importance for protein digestion in the intestinal lumen of fish due to the percent of inhibition that was registered was very weak for different fish species (Dimes et al., 1994; Izvekova and Solovyev, 2016). It has been shown, based on inhibitory analyses, that the input of metallo-proteases is relatively low and does not exceed 10% (Lazo et al., 2007; Chakrabarti et al., 2006a) but, for example, for *S. aurata* the input of metallo-proteases was similar with trypsin and chymotrypsin (Alarcón et al., 1998).

5. Conclusions

These results indicate that arriving at conclusions about the digestive capacity of fish may vary depending on the methodological (correlation analysis and/or inhibitor analysis) and stage of development considered (mainly based on inhibitor analysis). Moreover, correlation analysis as shown in this meta-analysis, may be used as an integrative biomarker and has demonstrated the relative importance of trypsin, or chymotrypsin, or both of them for the proper assessment of digestive capacity at early life stages of fish, as well as a tool for the proper formulation of compound feeds for fish species of interest. Theoretically, this approach is also appropriate for estimation of relative inputs of trypsin and chymotrypsin in any experiments where series of digestive enzyme activity measurements are enough for running correlation analyses. As the bonds cleaved by trypsin and chymotrypsin in proteins and polypeptides are distinct, inclusion of appropriate components in fish diet will potentially increase the feed efficiency.

Author contributions

Conceptualization, M.S. and E.G.; Data Curation, M.S. and E.G.; Formal Analysis, M.S., E.K., and E.G.; Funding Acquisition, M.S., E.G., and E.K.; Investigation, M.S., E.K., and E.G.; Methodology, M.S., and E.G.; Resources, M.S., E.K., and E.G.; Writing—Original Draft, M.S., E.K., and E.G.; Writing—Review and Editing, M.S., E.K., and E.G. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

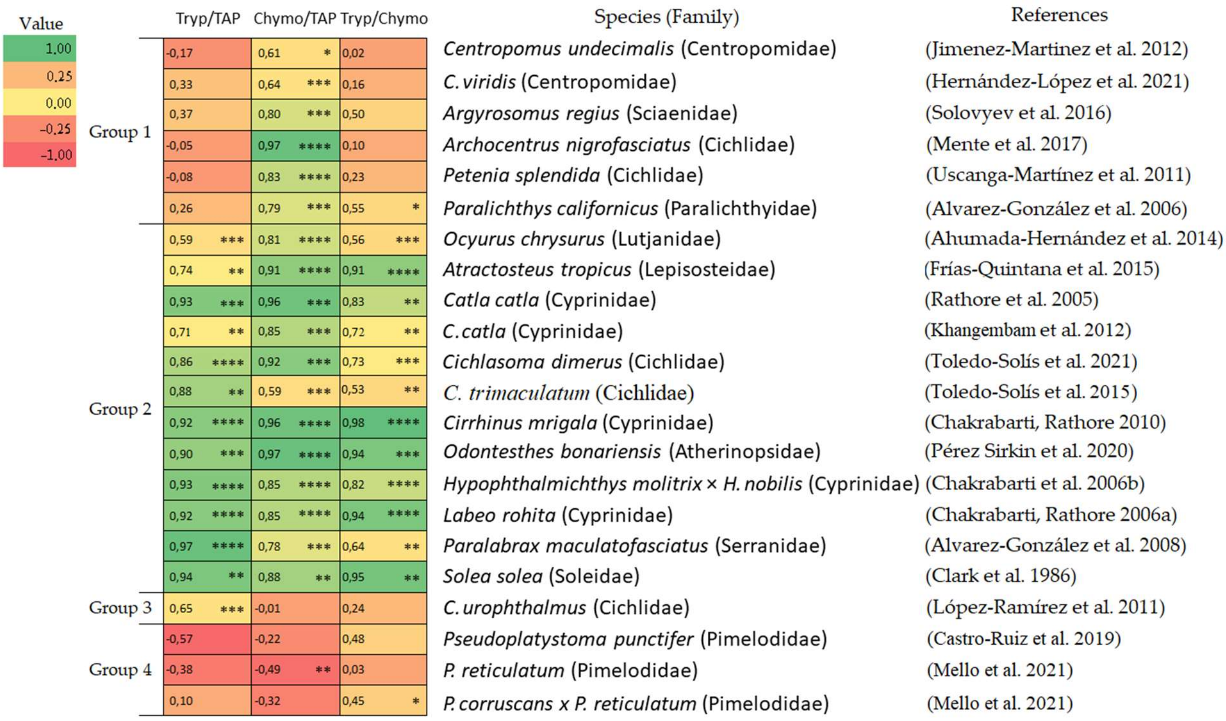

Fig. 1. Heat map based on *r* values calculated with Pearson correlation analysis among the activity of trypsin (Tryp), chymotrypsin (Chymo), and total alkaline proteases (TAP) during ontogenetic development of different fish species. Correlation coefficients that were statistically significant ($p < 0.1$) are marked by white asterisks. Tryp – trypsin, Chymo – chymotrypsin, TAP – total alkaline proteases. The asterisks denote ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$, *$p < 0.10$.

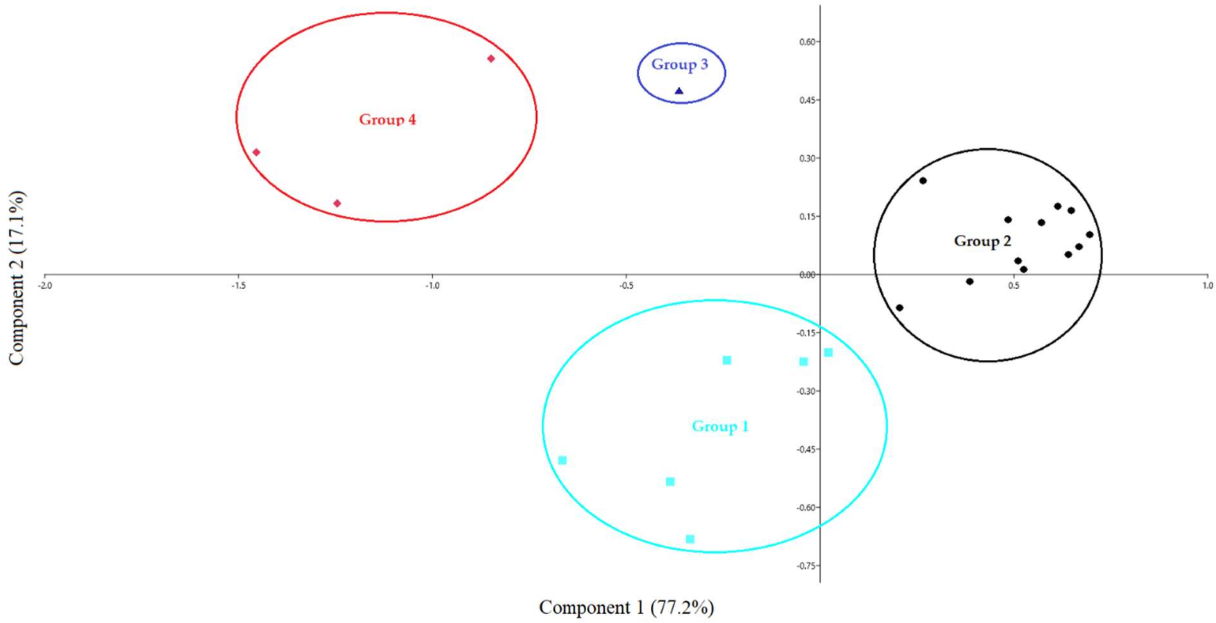

Fig. 2. Principal Components Analysis (PCA) based on correlation coefficients between trypsin and total alkaline proteases, chymotrypsin and total alkaline proteases, trypsin and chymotrypsin.

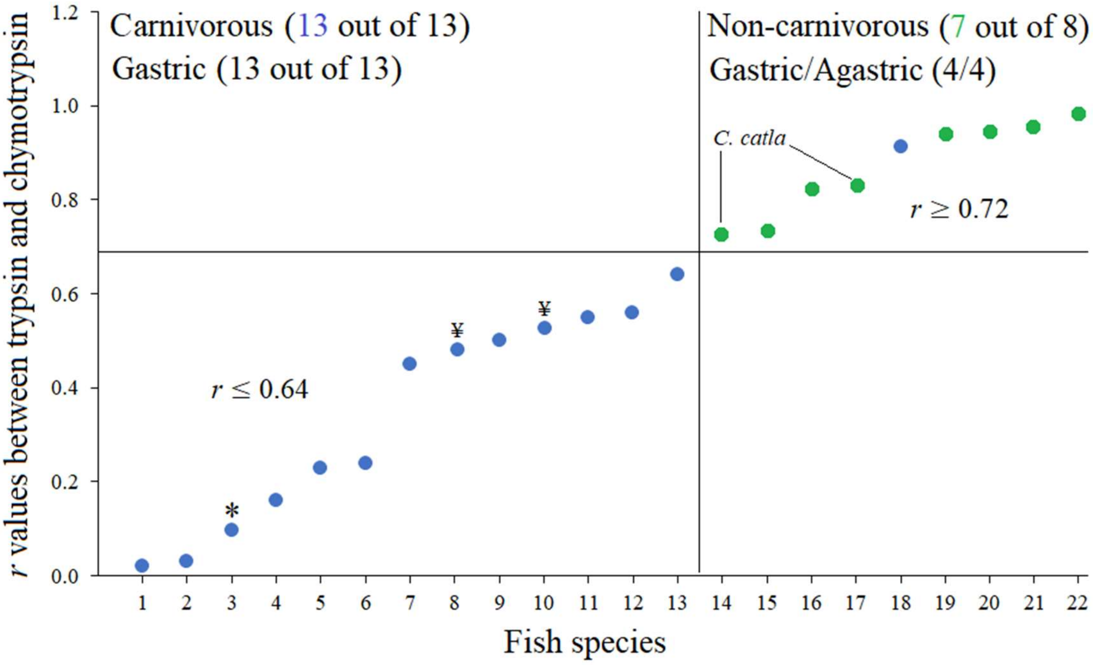

Fig. 3. Pearson correlationship (*r* values) between trypsin and chymotrypsin for studied fish species. 1. *Centropomus undecimalis* (Jimenez-Martinez et al., 2012), 2. *Pseudoplatystoma reticulatum* (Mello et al., 2021), 3. *Archocentrus nigrofasciatus* (Mente et al., 2017), 4. *C. viridis* (Hern´andez-L´opez et al., 2021), 5. *Petenia splendida* (Uscanga-Martínez et al., 2011), 6. *Cichlasoma urophthalmus* (L´opez-Ramírez et al., 2011), 7. *P. corruscans* × *P. reticulatum* (Mello et al., 2021), 8. *P. punctifer* (Castro-Ruiz et al., 2019), 9. *Argyrosomus regius* (Solovyev et al., 2016), 10. *C. trimaculatum* (Toledo-Solís et al., 2015), 11. *Paralichthys californicus* (Alvarez-Gonz´alez et al., 2006), 12. *Ocyurus chrysurus* (Ahumada-Hern´andez et al., 2014), 13. *Paralabrax maculatofasciatus* (Alvarez- Gonz´alez et al., 2008), 14. *Catla catla* (Khangembam et al., 2017), 15. *C. dimerus* (Toledo-Solís et al., 2021Toledo-Solís et al., 2021), 16. *Hypophthalmichthys molitrix* × *H. nobilis* (Chakrabarti et al., 2006b), 17. *C. catla* (Rathore et al., 2005), 18. *Atractosteus tropicus* (Frías-Quintana et al., 2015), 19. *Odontesthes bonariensis* (P´erez Sirkin et al., 2020), 20. *Labeo rohita* (Chakrabarti et al., 2006a), 21. *Solea solea* (Clark et al., 1986), 22. *Cirrhinus mrigala* (Chakrabarti and Rathore, 2010); Blue circle – generally carnivorous species, green circle – generally non-carnivorous species.

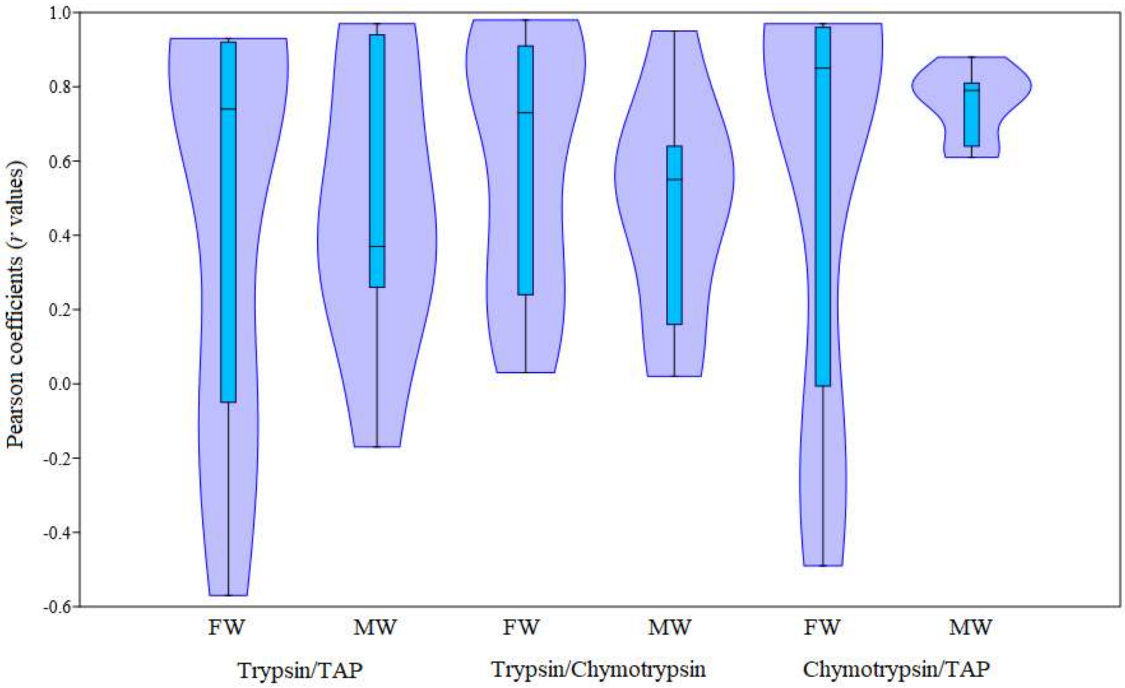

Fig. 4. A Violin plot for Pearson correlationship (*r* values) between chymotrypsin/total alkaline proteases (TAP), trypsin/chymotrypsin, and trypsin/total alkaline proteases for studied marine and freshwater fishes. Abbreviations: MW: marine fish species, FW, freshwater fish species. Box plots designate mean, standart error, and 95% confidence interval of the data.

1 ***Centropomus undecimalis***

Jimenez-Martinez et al. 2012
Digestive enzymes activities during early ontogeny in common snook (*Centropomus undecimalis*)
Fish Physiol. Biochem. 38 (2), 441–454

Enzymatic activity	**DAH***								
	0	**1**	**4**	**5**	**7**	**12**	**30**	**34**	**36**
Trypsin	0,033	0,035	0,037	0,037	0,05	0,052	0,033	0,028	0,029
Chymotrypsin	1	1	2,3	2,5	2,6	2,6	3,92	3,5	2
Total alkaline proteases	1230	1234,2	3461,5	2100	393,4	6729	5990	6000	5800

DAH* - day after hatching

2 ***Centropomus viridis***

Hernández-López et al. 2021
Characterization of digestive enzymes during early ontogeny of white snook (*Centropomus viridis*)
Aquaculture. 535, 736399

Enzymatic activity	**DAH**																		
	1	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**	**21**	**23**	**25**	**27**	**29**	**31**	**33**	**35**	**40**
Trypsin	18	14	5	10	8	14	11	8	9	13	15	18,5	15	22	12	13	10	23	17
Chymotrypsin	5	29	10,5	8	2	8	53	17	9	10	8	15	12	28	11	13	12,5	15	24
Total alkaline proteases	10	11	7	12	6	12	17	11	10	10	9	12	5	13	11,5	12,5	5,5	11	17

3 ***Argyrosomus regius***

Solovyev et al. 2016
Morphological and functional description of the development of the digestive system in meagre
Aquaculture. 464, 381–391

Enzymatic activity	**DAH**									
	1	**5**	**9**	**12**	**20**	**25**	**30**	**38**	**45**	**50**
Trypsin	0,1	2,2	0,3	0,48	1,1	1,3	0,28	0,5	0,08	0,48
Chymotrypsin	0,11	0,24	0,22	0,1	0,13	0,052	0,02	0,02	0,03	0,01
Total alkaline proteases	0,01	2,1	3,8	0,3	1,1	1,2	0,2	0,3	0,01	0,3

4 ***Archocentrus nigrofasciatus***

Mente et al. 2017
Digestive enzyme activity during initial ontogeny and after feeding diets with different protein sources in zebra cichlid, *Archocentrus nigrofasciatus*
J. World Aquac. Soc. 48 (5), 831–848

Enzymatic activity	**DAH**								
	0	**3**	**7**	**10**	**16**	**20**	**23**	**26**	**30**
Trypsin	0,051	0,1	0,08	0,1	0,082	0,075	0,052	0,048	0,036
Chymotrypsin	0,8	1	1,5	1,7	2,57	2	1,55	1,95	1,5
Total alkaline proteases	0,004	0,006	0,02	0,025	0,047	0,038	0,028	0,03	0,027

5 ***Patenia splendida***

Uscanga et al. 2011
Changes in digestive enzyme activity during initial ontogeny of bay snook *Patenia splendida*
Fish Physiol. Biochem. 37 (3), 667–680

Enzymatic activity	**DAH**																		
	0	**2**	**4**	**9**	**11**	**12**	**14**	**16**	**18**	**21**	**24**	**26**	**30**	**32**	**36**	**39**	**42**	**44**	**60**
Trypsin	0	1	1	0	1	0	0	1	2	2,5	2,5	36	17	8	10	7	18	40	300
Chymotrypsin	0	0	0	30	50	40	10	12	38	2	40	160	420	295	320	200	210	260	190
Total alkaline proteases	0	0	0	30	50	25	20	19	25	20	25	40	170	140	160	55	25	30	20

6 ***Paralichthys californicus***

Alvarez-González et al. 2006
Development of digestive enzymes in California halibut *Paralichthys californicus* larvae
Fish Physiol. Biochem. 31, 83–93

Enzymatic activity	**DAH**										
	0	**1**	**3**	**4**	**5**	**8**	**12**	**15**	**18**	**25**	**30**
Trypsin	4	3,5	2	5,4	8	6,5	4	4,4	5	4	3,8
Chymotrypsin	0	0	210	290	300	220	180	190	240	130	20
Total alkaline proteases	0	0,2	3,98	4	4,45	2,2	2,4	3,95	3,9	4,45	2,2

7 ***Ocyurus chrysurus***

Ahumada-Hernández et al. 2014
Changes of digestive enzymatic activity on yellowtail snapper (*Ocyurus chrysurus*) during initial ontogeny
Int. J. Biol. 6 (4), 110–118

Enzymatic activity	**DAH**																				
	1	**2**	**3**	**4**	**5**	**7**	**8**	**10**	**12**	**15**	**17**	**19**	**21**	**22**	**26**	**28**	**30**	**32**	**34**	**38**	**42**
Trypsin	0	0,1	0	0	20	210	220	300	0	190	400	100	390	370	210	490	410	620	350	210	780

Chymotrypsin	0	0	0	0	0,2	0	4	2	0	2	17	17	5	11	9	18	4,5	19	6	2	5
Total alkaline proteases	0	2	0	0	6	20	18	18	40	50	230	360	60	200	230	590	190	500	210	410	220

8 ***Atractosteus tropicus***

Frías-Quintana et al. 2015
Development of digestive tract and enzyme activities during early ontogeny of the tropical gar *Atractosteus tropicus*
Fish Physiol Biochem. 41 (5), 1075–1091

	DAH								
Enzymatic activity	**0**	**4**	**5**	**6**	**9**	**15**	**20**	**25**	**32**
Trypsin	0	0	0,01	0,008	0,01	0,01	0,034	0,055	0,062
Chymotrypsin	0,9	0,6	0,8	0,8	0,7	1,2	1,7	1,7	3
Total alkaline proteases	0,9	0,8	0,5	0,85	0,6	0,6	1,3	1,2	5,1

9 ***Catla catla***

Rathore et al. 2005
Digestive enzyme patterns and evaluation of protease classes in *Catla catla* (Family: Cyprinidae) during early developmental stages
Comp. Biochem. Physiol. B. 142 (1), 98–106

	DAH					
Enzymatic activity	**4**	**12**	**20**	**22**	**24**	**34**
Trypsin	53,55	12,03	34,85	33,56	64,92	118,07
Chymotrypsin	57,63	250	497,3	549,4	984,58	1500
Total alkaline proteases	286,96	240	450	527,67	1100,18	2200

10 ***Catla catla***

Khangembam et al. 2017
Effect of cortisol and triiodothyronine bath treatments on the digestive enzyme profile and growth of *Catla catla* larvae during ontogenic development
Aquac. Res. 48 (5), 2173–2185

	DAH										
Enzymatic activity	**5**	**8**	**11**	**14**	**17**	**20**	**23**	**26**	**29**	**32**	**35**
Trypsin	20	18	50	25	27	90	18	50	110	100	110
Chymotrypsin	10	50	30	40	70	75	120	130	200	420	250
Total alkaline proteases	50	70	90	100	100	90	100	120	500	1700	2100

11 ***Cichlasoma dimerus***

Toledo-Solís et al. 2021
Changes in digestive enzyme activities during the early ontogeny of the South American cichlid (*Cichlasoma dimerus*)
Fish Physiol. Biochem. 47 (4), 1211–1227

	DAH										
Enzymatic activity	**0**	**1**	**3**	**6**	**9**	**11**	**13**	**15**	**17**	**19**	**21**
Trypsin	0	0	0	0	0	0	0,05	0,07	0,15	0,11	0,14
Chymotrypsin	0	0	0	0,26	0,02	0,14	0,31	0,305	0,31	0,29	0,26
Total alkaline proteases	0	0	25	60	10	24	50	75	90	85	80

12 ***Cichlasoma trimaculatum***

Toledo-Solís et al. 2015
Changes on digestive enzymes during initial ontogeny in the three-spot cichlid *Cichlasoma trimaculatum*
Fish Physiol Biochem. 41 (1), 267–279

	DAH																			
Enzymatic activity	**0**	**1**	**3**	**6**	**9**	**11**	**13**	**15**	**17**	**19**	**21**	**24**	**27**	**30**	**33**	**36**	**39**	**42**	**45**	**60**
Trypsin	0	0	0,05	0,1	0,2	0,15	0,25	1,6	1,7	1,3	0,8	0,45	0,65	0,2	0,27	0,25	0,27	0,3	0,29	0,2
Chymotrypsin	0	0,03	0	0,31	0,5	0,48	0,75	0,77	0,78	0,76	0,7	0,72	0,775	0,79	0,773	0,725	0,795	0,795	0,35	0,19
Total alkaline proteases	0	0	0	0,13	0,15	0,14	0,13	0,27	0,46	0,33	0,21	0,215	0,3	0,07	0,05	0,06	0,09	0,11	0,07	0,05

13 ***Cirrhinus mrigala***

Chakrabarti and Rathore 2010
Ontogenic changes in the digestive enzyme patterns and characterization of proteases in Indian major carp *Cirrhinus mrigala*
Aquac. Nutr. 16 (6), 569–581

	DAH															
Enzymatic activity	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**	**22**	**24**	**26**	**28**	**30**	**32**	**34**
Trypsin	20	10	10	10	15	30	90	100	240	250	510	730	620	590	510	500
Chymotrypsin	0	0	0	0	0	200	1500	1600	2000	3000	4000	7000	6900	6800	6100	5600
Total alkaline proteases	0	0	10	70	100	800	820	1010	1050	1700	2000	2980	3900	3800	4000	4250

14 ***Odontesthes bonariensis***

Pérez Sirkin et al. 2020
Digestive enzyme activities during pejerrey (*Odontesthes bonariensis*) ontogeny
Aquaculture. 524 (6), 735151

	WPH**						
Enzymatic activity	**1**	**2**	**3**	**4**	**5**	**7**	**9**
Trypsin	17,76	15	16	12	13,55	57,18	23,84

WPH - weeks post hatching**

Chymotrypsin	10	0	20	30	500	1724	454
Total alkaline proteases	10	5	20	30	1000	3000	1500

15 ***Hypophthalmichthys molitrix × H. nobilis***

Chakrabarti et al. 2006b
Functionak changes in digestive enzymes and characterization of proteases of silver carp (male) and bighead carp (female) hybrid...
Aquaculture. 253, 694–702

	DAH															
Enzymatic activity	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34
Trypsin	10	12	28	21	22	23	23	23	30	31	25	38	43	50	70	59
Chymotrypsin	0	0	0	18	20	25	26	55	210	430	430	380	410	400	530	500
Total alkaline proteases	0	10	20	70	80	70	80	80	130	180	220	290	580	610	780	500

16 ***Labeo rohita***

Chakrabarti et al. 2006a
Study of digestive enzyme activities and partial characterization of digestive proteases in a freshwater teleost, Labeo rohita, during early ontogeny
Aquac. Nutr. 12 (1), 35–43

	DAH															
Enzymatic activity	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**	**22**	**24**	**26**	**28**	**30**	**32**	**34**
Trypsin	20	15	0	0	5	10	15	50	55	100	180	160	165	270	300	350
Chymotrypsin	0	0	0	10	15	30	35	70	250	260	500	770	760	730	950	1750
Total alkaline proteases	0	10	0	50	70	110	220	220	500	800	970	1200	1270	1580	1210	1270

17 ***Paralabrax maculatofasciatus***

Alvarez-González et al. (2008)
Development of digestive enzyme activity in larvae of spotted sand bass *Paralabrax maculatofasciatus*. 1. Biochemical analysis.
Fish Physiol. Biochem. 34 (4), 373–384

	DAH												
Enzymatic activity	**0**	**1**	**2**	**3**	**4**	**5**	**7**	**9**	**12**	**15**	**18**	**25**	**30**
Trypsin	0,5	1	0,6	1,8	1,75	1	0,8	3,9	10	8	8,5	2	1,5
Chymotrypsin	140	150	135	330	250	300	340	370	339	375	360	210	170
Total alkaline proteases	1	0	2	15	17	9	10	27	50	42	45	7	3

18 ***Solea solea***

Clark et al. 1986
Protease development in dover sole [*Solea solea* (L.)]
Aquaculture. 53 (3-4), 253–262

	Days				
Enzymatic activity	**24**	**49**	**80**	**200**	**Adult**
Trypsin	1,76	3	8	10	14
Chymotrypsin	11,3	15	21	39	42
Total alkaline proteases	0,5	2	2,3	2,8	4,5

19 ***Cichlasoma urophthalmus***

López-Ramírez et al. 2011
Development of digestive enzymes in larvae of Mayan cichlid *Cichlasoma urophthalmus*
Fish Physiol. Biochem. 37 (1), 197–208

	DAH																		
Enzymatic activity	**0**	**2**	**6**	**8**	**11**	**12**	**14**	**18**	**20**	**22**	**24**	**28**	**30**	**32**	**34**	**38**	**42**	**45**	**60**
Trypsin	0	0	0,00001	0,00002	0,00003	0,00018	0,0001	0,00008	0,00022	0,00026	0,0006	0,0003	0,00024	0,00021	0,00018	0,0002	0,00023	0,0004	0,00016
Chymotrypsin	0,001	0,003	0	0,001	0,0018	0,0063	0,0056	0,0058	0,0054	0,008	0,0078	0,0076	0,0056	0,011	0,0054	0,0045	0,058	0,0078	0,0024
Total alkaline proteases	0	0	0	0	10	87	90	72	74	89	95	80	74	80	85	28	20	66	28

20 ***Pseudoplatystoma punctifer***

Castro-Ruiz et al. 2019
Ontogeny of the digestive enzyme activity of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer* (Castelnau, 1855)
Aquaculture. 504, 210–218

	DAH						
Enzymatic activity	**0**	**4**	**12**	**17**	**20**	**25**	**27**
Trypsin	0,007	0,0055	0,005	0,0075	0,0065	0,009	0,011
Chymotrypsin	0,22	0,06	0,07	0,1	0,12	0,1	0,17
Total alkaline proteases	0,78	0,76	1,5	0,2	0,18	0,4	0,38

21 ***Pseudoplatystoma reticulatum***

Mello et al. 2021
Ontogeny of the digestive system and the profile of proteases in larvae of cachara (*Pseudoplatystoma reticulatum* Siluriformes: Pimelodidae) and its hybrid (*Pseudoplatystoma corruscans* x *Pseudoplatystoma reticulatum*)
J. Fish Biol. 99 (3), 1135–1139

	Hours						DAH											
Enzymatic activity	**0**	**8**	**13**	**24**	**32**	**40**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**15**	**20**	**25**
Trypsin	0,025	0,022	0,03	0,031	0,035	0,026	0,024	0,022	0,023	0,024	0,03	0,02	0,024	0,019	0,024	0,024	0,024	0,023

Chymotrypsin	0,038	0,04	0,015	0,022	0,022	0,021	0,027	0,013	0,015	0,02	0,012	0,02	0,019	0,01	0,012	0,011	0,015	0,019
Total alkaline proteases	1,8	1,6	0,2	1,9	2,1	3	4	6	10	20	15	17	18	90	130	70	50	91

22 *P. corruscans × P. reticulatum*

Mello et al. 2021

Ontogeny of the digestive system and the profile of proteases in larvae of cachara (*Pseudoplatystoma reticulatum* Siluriformes: Pimelodidae) and its hybrid (*Pseudoplatystoma corruscans* x *Pseudoplatystoma reticulatum*)

J. Fish Biol. 99 (3), 1135–1139

	Hours						**DAH**											
Enzymatic activity	**0**	**8**	**13**	**24**	**32**	**40**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**15**	**20**	**25**
Trypsin	0,025	0,027	0,024	0,028	0,042	0,035	0,024	0,022	0,023	0,04	0,025	0,019	0,023	0,025	0,024	0,025	0,0245	0,04
Chymotrypsin	0,015	0,023	0,015	0,024	0,03	0,027	0,028	0,013	0,019	0,012	0,012	0,011	0,014	0,015	0,011	0,019	0,01	0,019
Total alkaline proteases	0,9	0,9	0,2	1,9	2,2	3,5	3,9	5,5	12	30	12	25	23	140	130	120	110	170

Pearson correlation analysis (*r* and p-values) among the activity of trypsin (Try), chymotrypsin (Chy), and total alkaline proteases (AP) (p < 0.1) are in bold type and marked by red during ontogenetic development of different fish species. P-values are above, *r* values are below

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,96	0,66
Chymotrypsin	0,02		**0,083**
Total alkaline proteases	-0,17	**0,61**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,51	0,17
Chymotrypsin	0,16		**0,003**
Total alkaline proteases	0,33	**0,64**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,14	0,29
Chymotrypsin	0,50		**0,006**
Total alkaline proteases	0,37	**0,80**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,81	0,90
Chymotrypsin	0,10		**0,00002**
Total alkaline proteases	-0,05	**0,97**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,34	0,76
Chymotrypsin	0,23		**1,15E-05**
Total alkaline proteases	-0,08	**0,83**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		**0,08**	0,43
Chymotrypsin	**0,55**		**0,003**
Total alkaline proteases	0,26	**0,79**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		**0,008**	**0,005**
Chymotrypsin	**0,56**		**7,06E-06**
Total alkaline proteases	**0,59**	**0,81**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,0007	0,02
Chymotrypsin	0,91		0,0007
Total alkaline proteases	0,74	0,91	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,04	0,006
Chymotrypsin	0,83		0,002
Total alkaline proteases	0,93	0,96	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,012	0,014
Chymotrypsin	0,72		0,001
Total alkaline proteases	0,71	0,85	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,01	0,0007
Chymotrypsin	0,72		7,08E-05
Total alkaline proteases	0,86	0,92	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,02	2,47E-07
Chymotrypsin	0,53		0,0065
Total alkaline proteases	0,88	0,59	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		2,56E-11	5,82E-07
Chymotrypsin	0,98		2,57E-09
Alkaline proteases	0,92	0,96	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,002	0,005
Chymotrypsin	0,94		0,0002
Total alkaline proteases	0,90	0,97	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		**0,0001**	**1,18E-07**
Chymotrypsin	**0,82**		**3,83E-05**
Total alkaline proteases	**0,93**	**0,84**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		**4,06E-08**	**6,19E-07**
Chymotrypsin	**0,94**		**3,56E-05**
Total alkaline proteases	**0,92**	**0,85**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		**0,02**	**6,90E-08**
Chymotrypsin	**0,64**		**0,002**
Total alkaline proteases	**0,97**	**0,78**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		**0,015**	**0,019**
Chymotrypsin	**0,95**		**0,046**
Total alkaline proteases	**0,94**	**0,88**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,32	**0,003**
Chymotrypsin	0,24		0,98
Total alkaline proteases	**0,65**	-0,006	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,28	0,18
Chymotrypsin	0,48		0,63
Total alkaline proteases	-0,57	-0,22	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		0,91	0,12
Chymotrypsin	0,03		**0,039**
Total alkaline proteases	-0,38	**-0,49**	

	Trypsin	Chymotrypsin	Total alkaline proteases
Trypsin		**0,06**	0,71

Chymotrypsin	**0,45**		0,20
Total alkaline proteases	0,10	-0,32	