Understanding the early ontogenetic stages of *Mugil liza* (Mugilidae): morphological traits and digestive/metabolic profile of pre-juveniles after recruitment

Running Title: Digestive traits of *Mugil liza*

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Abstract

The family Mugilidae Family consists mainly of diadromous species, whose reproduction occurs in offshore waters. Pre-juveniles shift their diet in the surf zone (zooplanktophagous to iliophagous). Later, during their recruitment into estuaries, huge changes take place in their digestive system. However, digestive and metabolic characteristics and some morphological traits at recruitment are unknown for Mugilidae. We performed comparative studies on early and late pre-juveniles of *Mugil liza* recruited in Mar Chiquita Coastal Lagoon (37°32′–37°45′S; 57°19′–57°26′W, Argentina). We determined digestive enzyme activities (intestine), energy reserves (liver/muscle), total/standard length, total weight, intestinal coefficient, hepatosomatic index and retroperitoneal fat. Pre-juveniles exhibited amylase, maltase, sucrase, lipase, trypsin and APN activities, which were maintained over a wide range of pH and temperature and exhibited Michaelis-Menten kinetics. In late pre-juveniles, amylase (422±131µmol maltose x min^{-1} x $mgprot^{-1}$), sucrase (86±14 mg glucose x min^{-1} x mg $prot^{-1}$), trypsin (84±9 µmoles x min^{-1} x mg $prot^{-1}$) and APN (0.58±0.08 µmoles x min^{-1} x mg $prot^{-1}$) activities were higher (42, 28, 35 and 28%, respectively) than in the early stage. Also, the intestinal coefficient was higher in late (3.04) than early (2.06) pre-juveniles. Moreover, liver appeared to be a main site of glycogen and triglyceride storage in late pre-juveniles, muscle being the site of storage in early pre-juveniles, which exhibited higher glycogen, free glucose and protein concentrations (92, 82, 32%, respectively). The results suggest that pre-juveniles of *M. liza*

exhibit an adequate digestive battery to perform complete hydrolysis of various dietary substrates, availability of energy reserves and morphological characteristics to support their feeding habit and growth after recruitment. Our results represent an important contribution to knowledge of the ecology and digestive physiology of pre-juveniles of Mugilidae in the wild.

Keywords: *Mugil liza*, Mar Chiquita Coastal Lagoon, pre-juveniles**, digestive enzymes, energy reserves, recruitment**

1 Introduction

Members of the family Mugilidae, generally known as mullets, are coastal marine fishes with a worldwide distribution. They inhabit offshore and coastal waters, but also spend part or even their whole life cycle in coastal lagoons, lakes and/or rivers (González-Castro and Ghasemzadeh, 2016; Nelson, 2006). They occupy a unique position in the food web, due to their primarily detritivorous (iliophagous) feeding (Blaber, 1976; Cardona, 2016) and possess economic importance, particularly the *Mugil cephalus* Linnaeus, 1758 species-complex (constituted by 14 parallel lineages that included *Mugil liza* Valenciennes, 1836 and 13 other lineages, all currently designated as *M. cephalus* (González-Castro and Ghasemzadeh, 2016; Whitfield et al., 2012)). The taxa belonging to the *M. cephalus*

species-complex constitutes the basis of significant commercial as well as artisanal fisheries around the world (Crosetti, 2016; González-Castro et al., 2009; Vieira et al., 2008). Mullets are also cultured in many regions of the world, both in extensive systems and in semi-intensive or intensive systems, often in polyculture with other species (Crosetti, 2016; Leber et al., 2016; Liao et al., 2016; Sadek, 2016).

The southern population of *Mugil liza* is distributed from Argentina (47 °S) to the state of São Paulo, Brazil (23 °S) (Mai et al., 2014). In Argentina it supports a small-scale fishery, with catches reported between 5.4 and 78.8 t from 2000 to 2010, with a maximum capture of 194.0 t in 2004 (González-Castro 2007; González-Castro et al., 2009a; Navarro et al., 2014).

Mar Chiquita Coastal Lagoon (MCh) is a brackish-water coastal lagoon located in the Buenos Aires Province (Argentina) (37°32′–37°45′S; 57°19′–57°26′W), considered since 1996 as a World Biosphere Reserve by UNESCO. *Mugil liza* represents the second most abundant species of mullet in MCh, in terms of both biomass and abundance (González-Castro et al., 2009a, b).

Mugil liza is characterized by a complex diadromous life cycle. Coastal reproductive spawning occurs from May/June until August, in the offshore waters between Santa Catarina and Paraná, Brazil (28-26 °S) (González-Castro and Minos, 2016; Lemos et al., 2014). In August-September the reproductive events end and neustonic post larvae are carried from offshore waters to the coast (surf zone), by wind generated surface currents

(González-Castro et al., 2009a; Vieira and Scalabrin, 1991). There, in the surf zone, the pre-juveniles (<30 mm TL) can stay for a variable period of time, changing from zooplanktophagous to iliophagous feeding, prior to recruitment into estuarine systems (Vieira, 1991; Acha, 1990).

During their recruitment into estuaries/coastal lagoons, young mullets (*M. liza*) undergo a series of changes in a short, but crucial period of time, which includes the morpho-anatomical adaptation to iliophagy and also to brackish waters (Castellini et al., 2019). This implies a strong challenge for the recruiters, with direct repercussions for the survival of the species resource. It is precisely at this moment, when fry are termed Querimana by some authors (Thomson, 1997; Castellini et al., 2019): i.e, pre-juvenile mullets having two anal spines, including the transition period until the third spine is completely developed (as in adult specimens), when it becomes juvenile (Jacot, 1920; Thomson, 1997). Castellini et al. (2019) analysed the daily age at recruitment of Querimana (pre-juveniles) inside MCh and recorded individuals between 67 and 117 days and 30 to 43 mm standard length. During this particular stage, young mullets undergo huge changes to their digestive system, in order to satisfy their new iliophagous feeding mode. Moreover, in a few days, pre-juvenile individuals will move from the coast (surf zone) to the inner zones of coastal-lagoons or estuaries. There, they must to adapt to live in a shallow environment, with salinities close to fresh water (ranging between 0-10) (González-Castro et al., 2009b). Despite the importance of pre-juveniles being successful for the establishment of the future

cohorts of juveniles, not much attention has been given to this ontogenetic stage. There are no published works dealing with both digestive and metabolic characteristics at the biochemical level (e.g. occurrence of specific digestive enzymes/presence and distribution of energy reserves) and also with morphological traits of recruited pre-juveniles in the wild.

The presence of specific digestive enzymes (carbohydrases/lipases/proteases) in the intestine (the main site of digestion and absorption of nutrients in fish) is generally related to the nature of the dietary items potentially used in metabolic processes (Karasov and Douglas, 2013; Sanz et al., 2015; Steimberg, 2018). The determination of amylase activity (essential in initial steps of the digestion of key carbohydrates), allows evaluation of the capacity for digestion of glycogenic substrates (Dhital et al., 2013; Karasov and Douglas, 2013). To determine the occurrence of lipase and key proteases such as trypsin allows assessing lipolytic and proteolytic digestive capacity (Bakker et al. 2011; Steimberg, 2018). Trypsin is an endoprotease, which is also one of the most important proteases in various teleost fish (Steimberg, 2018). Membrane-bound disacharidases (e.g. maltase and sucrase) and the ectopeptidase aminopeptidase-N (APN) play a main role in the final steps of digestion of glycogenic carbohydrates and proteins, respectively. Their existence in the intestine are an index of intestinal maturity and extracellular digestion (del Valle et al. 2016; Holt, 2011; Tran et al., 2011). The determination of energy reserves (glycogen, triglycerides, protein) in main storage organs is a common tool used to evaluate the

metabolic characteristics at the biochemical level of an individual. When it is performed concomitant with that of digestive enzymes, it allows an integrated analysis of the digestive and metabolic profile (del Valle and López Mañanes, 2012; del Valle et al., 2016). On the other hand, to know morphological traits further helps in understanding the relationship between physiological and biochemical functions of the digestive tract and links to ecological issues (Kalhoro et al., 2018).

In this context, the aim of this work was to comparatively analyse the digestive/metabolic characteristics at the biochemical level and morphological traits of early and late pre-juveniles of *M. liza* in the wild, after its recruitment into Mar Chiquita Coastal Lagoon (MCh), including: a) the total length, total weight, intestinal coefficient, hepatosomatic index (HIS) and retroperitoneal fat as parameters of physical conditions; b) the occurrence of amylase, maltase, sucrase, lipase, trypsin and APN activities in the intestine and c) the concentration of glycogen, free glucose, triglycerides and protein in energy storage sites. We hypothesized that early and late pre-juveniles of *M. liza* exhibit differences in their digestive and metabolic characteristics at the biochemical level and morphological traits, which constitute adaptive strategies to support feeding habit and growing after recruitment in Mar Chiquita Coastal Lagoon.

2 Materials and methods

2.1 Study area

Pre-juvenile individuals were collected during August 2019 after their recruitment into the Zone II of MCh, according to González-Castro et al. (2009b). The Zone II is located inside MCh, 6 Km far from the mouth of the lagoon to the sea and is characterized by mixo-mesohaline waters ranging in salinity from 5 to 35. Water temperature ranges between 6°C (winter) and 21°C (summer). It is a shallow environment, approximately between 0.30 and 1.20 m in depth.

2.2 Collection of individuals and sample procedures

Pre-juvenile individuals were collected with a beach seine net (10 m in length × 1.8 m high; each wing measured 4 m in length and the cod-end was 3 m in length; the mesh in the lateral wings was 10 mm, and the mesh in the cod-end was 5 mm) (Cousseau et al., 2001). Water temperature (13°C) and salinity (18 PSU) data were recorded using an alcohol thermometer and a refractometer, respectively. Fish were taxonomically identified according to Cousseau and Perrotta (2013) and González-Castro et al. (2012). Fresh samples were put immediately on ice until loss of consciousness (about 10 min), frozen and subsequently processed at the laboratory (Albanesi 2018; Albanesi et al 2017, 2018; del Valle et al., 2016; Ma et al; 2019). Total length (TL), standard length (SL) and total weight (TW) of each mullet were recorded, employing a digital caliper to the nearest mm

and an electronic balance (0.1 g). The mullet sample was grouped into “early” and “late” pre-juveniles (n=10 for each stage), according to the following criterion: i) early pre-juveniles refers to young mullets, between 23 and 33 (mm) SL, with two anal spines and the third anal element yet segmented, but with signs of incipient hyalinization; ii) late pre-juveniles refers to those young individuals, between 38 and 47 (mm) SL, where the third anal spine undergo hyalinization and is almost completely developed, but a slightly segmentation in it is still present. Immediately, intestine, liver and muscle were excised for each individual, weighed and put on ice to be used to prepare homogenates and to determine hepatosomatic index (HSI %) (liver weight/total weight) x 100) and intestinal coefficient (intestinal length/standard length). The presence/absence of retroperitoneal fat was also recorded. The intestine and liver were separately homogenized in 50 mM Tris/HCl, pH 7.4 (4 ml of tissue g^{-1}) (CAT 9 120 homogenizer, T10 tool) on ice (Albanesi, 2018; del Valle et al., 2016). The same procedure was followed for the body muscle although 8 ml of tissue g^{-1} was used (Albanesi, 2018; del Valle et al., 2016). Due to the small size of the liver of early pre-juveniles, no homogenates could be prepared even by pooling organ from different individuals.

This study was conducted following the regulations and statements of Ethics Committee CICUAL (OCA 1499/12; FCEyNat, UNMdP, Argentina). There was no experimentation.

2.3 Biochemical assays

Amylase activity in intestine was measured using starch (15 mg mL^{-1}) as substrate, according to del Valle et al. (2016), Albanesi (2018), Asaro et al. (2018). Briefly, the corresponding sample was incubated for 15 min at 30 °C, in the presence of starch in 50 mM phosphate buffer (pH 7.4); 300 ul of dinitro salicylic acid reagent (Miller, 1959) was added for further incubation for 10 min at 100 °C. After cooling immediately on ice, the released maltose was assessed reading absorbance at 540 nm. To study the effect of pH, temperature and starch concentration, the activity was assayed at varying pH (5.2-8.2) (50 mM phosphate buffer), temperature (4-45°C) and starch concentration (0.06-18 mg x ml^{-1}) in the reaction mixture.

Maltase and sucrase activity were determined measuring the glucose released from the specific substrate as we detailed (Albanesi 2018; Asaro et al 2018; del Valle et al., 2016). The sample was incubated during 10 min at 30 °C with 42 mM of maltose or sucrose in buffer 0.1 M malate buffer (pH 6, 4). The reaction was stopped with 1.5 mL of a glycemia kit ((glucose oxidase 10 kU L; peroxidase 1 kU; l,4-aminophenazone 0.5 mmol L^{-1}; phosphates pH 7.0100 mmol L^{-1}, hydroxybenzoate12 mmol L^{-1}) (Wiener Lab Glicemia AA) and further incubated during 5 min at 37 °C. Glucose amount was quantified reading absorbance at 505 nm of the colored quinone complex. The effect of pH, temperature and substrate concentration on disaccharidases activity was studied measuring the activity at various pH **(**maltase: 3.5-8.0, sucrase 5.2-8.0) (0.1 mM maleate buffer), temperatures (4-

45°C) and substrate concentrations (0.56-42 mM) in the reaction mixture (del Valle et al., 2016).

Lipase activity was determined colorimetrically by quantification of p-nitrophenol (pNP) released from p-nitrophenyl-palmitate as we described (Michiels et al., 2013, del Valle et al., 2016). The sample was incubated with 0.85 mM pNPP in buffer 50 mM Tris-HCl pH 8.5 during 5 min at 37 ° C. The reaction was stopped by adding 0.5 ml of TCA 0.1% (w / v). The amount of p- nitrophenol released was determined by reading the absorbance at 410 nm. To determinate the effect of pH, temperature and substrate, the activity was assayed at various pH (6.0-9.0) (50 mM phosphate buffer, pH 6.0, 50 mM Tris-HCl buffer, pH 7.2-9.0), temperature (4-45°C) and pNPP concentrations (0.017-0.9 mM) of the reaction mixture.

Trypsin activity was determined using N-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA) as substrate (Candiotto et al., 2017) as we described (del Valle et al., 2016; Michiels et al., 2017). The sample was incubated in 1.23 mM of the substrate in 50 mM Tris buffer / HCl pH 9.0/ 400 mM Cl_2Ca during 15 min at 45 ° C. The reaction was stopped by adding 250µl of 0.1 M KOH and absorbance was measured at 405 nm. The effect of pH, temperature and substrate was determined by assaying the activity at various varying pH (range 6.0-11.0) (50 mM phosphate buffer, pH 6.0, 50 mM Tris-HCl buffer pH 7.4-9.0, 50 mM Glicine pH 11), temperatures (4-70°C) and BAPNA concentrations (0.12-1.23 mM) in the reaction mixture (del Valle et al., 2016).

The measurement of APN activity was made by using L-alanine-p-nitroanilide (L-Ala pNA) according to (Michiels et al., 2015a, 2017; del Valle et al., 2016). The reaction was initiated by the addition of substrate (final concentration 0.33mM) to a reaction mixture containing the sample in 50 mM Tris-HCl buffer to pH 7.4. After incubation for 15 minutes at 45 ° C, the reaction was stopped with 0.2 ml of cold 2 M acetic acid and absorbance was determined at 384 nm. To study the effect of pH and temperature on APN activity, the activity was measured at varying pH (range 6.0-9.0) **(**50 mM phosphate buffer, pH 6.0, 50 mM Tris-HCl buffer pH 7.4-9.0**),** temperature (4-45°C) and L-Ala-pNA concentrations (0.04-0.4 mM) in the reaction mixture (del Valle et al., 2016).

Glycogen was measured as glucose equivalent as previously described (Pinoni et al., 2011, 2013; del Valle et al., 2016). The corresponding sample was boiled for 4 minutes and then incubated in acetate buffer (pH 4.8) in the presence and absence of 0.2 mg ml-1 of α-amyloglucosidase (Sigma Chemicals) for 2.5 h at 55 °C. After incubation, samples were centrifuged at 3000 rpm for 15 minutes. Glucose was quantified in the supernatant using the commercial kit for enzyme glycemia (Wiener Lab AA). Released glucose from glycogen hydrolysis was calculated as the difference between the values obtained with and without α-amyloglucosidase. Free glucose was assessed from assay without α-amyloglucosidase.

Triglycerides (TAG) were measured by the colorimetric method of glycerol phosphate oxidase (TAG Wiener-Lab AA code 861110001). An aliquot of the corresponding sample was incubated with this reactant for 5 minutes at 37 °C (Michiels et al., 2016; Pinoni et al.,

2011). The amount of released glycerol was determined by reading the absorbance at 505 nm of the colored quinone complex.

The protein concentration was assayed according to Bradford (1976). Bovine serum albumin (0.96 mg x ml^{-1}) was used as standard.

2.4 Statistical analysis

The results of the effect of different substrate concentrations on the enzymatic activities were analysed by a nonlinear regression analysis (GraphPad Prism4.0 software). The curve that appears is the one that best fits to the experimental data according to estimation by GraphPad Prism 4.0 software, showing adjustment to Michaelis-Menten model. Km value (Michaelis–Menten constant) was estimated from this curve (GraphPadPrism 4.0 software). Statistical analysis was carried out using the Sigma 3.0 program for Windows, which automatically performs previous test of equality of variances and normality. Analysis of variance (one-wayANOVA) or t-test were used to estimate the statistical significance of the differences and $P<0.05$ was considered significant. ANOVA (Student-Newman-Keuls) was used to identify differences. (del Valle et al., 2016).

3 Results

3.1 Morphological traits of early and late pre-juveniles of *M. liza*

Early and late pre-juveniles exhibited statistically significant variations ($p<0.05$) in size, ranging between 26 and 40.5 mm TL (mean 36.08 mm) and 45 to 56 mm TL (mean 50.8 mm), respectively (Table 1). Moreover, they showed significant differences ($p<0.05$) in TW (mean of 0.34 and 1.74 g, respectively) and IC (2.06 and 3.04, respectively). However, no difference was found in hepatosomatic index of both sub-stages. In addition, retroperitoneal fat was not found in either early or late pre-juveniles (Table 1).

3.2 Digestive enzyme activities in intestine of early and late pre-juveniles of *M. liza*

Initially, we determined the occurrence and made a partial characterization of amylase, maltase, sucrase, lipase, trypsin and APN activities in intestine of late pre-juveniles. Amylase activity increased from pH 5.2 to 7.4 At pH 8.2 the activity decreased until values similar to those found at pH 5.2-6.0 (Fig. 1a). Amylase activity was similar at 4 and 20°C, but it enhanced at 30°C. At 45°C, the activity decreased being similar to that at 4 and 20°C (Fig. 1b). Amylase activity exhibited Michaelis-Menten kinetics (apparent Km= 3.58 mM) (Fig 1.c). Maltase activity increased from pH 5.2 to 6.4 and it was quite similar up to pH 8.0 (Fig. 1d). Maltase activity was similar within the range of temperature 4–45 °C (Fig. 1e) and exhibited Michaelis-Menten kinetics (apparent Km=2.60 mM) (Fig. 1f). Sucrase activity increased from pH 5.2 to 6.4. At pH 7.4 and 8.0 the activity was decreased being similar to that at pH 5.2 (Fig 1g). Sucrase activity was similar within the range of temperature 4–45 °C (Fig. 1g) and showed Michaelis-Menten kinetics (apparent Km=2.95

mM) (Fig. 1i). Lipase activity in intestine of late pre-juveniles was similar within the range of pH 6.0-9.0 (Fig. 2a); it increased from 4 to 45°C (Fig. 2b) and also exhibited Michaelis-Menten kinetics (apparent Km=0,58 mM) (Fig. 2c). Trypsin activity was detected over a wide range of pH. The activity increased from pH 7.4 to 9.0 and decreased at pH 11.00 to values similar to those at pH 7.4 (Fig. 2d). Trypsin activity was similar at 4 and 20ºC and increased at 45-75ºC (Fig. 2e) and showed a Michaelis-Menten kinetics (apparent Km= 1.38 mM) (Fig. 2f). APN activity increased from pH 6.0 to 7.4-9.0 (Fig. 2g), it was similar at 4 and 20ºC and increased with an enhancement of temperature up to 45°C (Fig. 2g). This activity also showed a Michaelis-Menten kinetics (apparent Km= 0.32 mM) (Fig. 2i).

The intestine of early pre-juveniles also exhibited amylase, maltase, sucrase, lipase, trypsin and APN activity (Fig. 3). Amylase, sucrase, trypsin and APN specific activity were significantly lower ($p<0.05$) when compared to the corresponding activity in late pre-juveniles (Fig. 3).

3.3 Energy reserves in early and late pre-juveniles of *M. liza*

Glycogen was detected in both liver and muscle in late pre-juveniles. Its concentration was higher in liver (about 87%) than in muscle (Fig. 4a). Free glucose concentration was similar in liver and muscle (Fig. 4b). Triglyceride concentration was also significantly

higher ($p<0.05$) in liver (about 87%) than in muscle (Fig. 4c). Protein concentration was significantly higher ($p<0.05$) in muscle (about 29%) than in liver (Fig. 4d).

Glycogen was also detected in muscle of early pre-juveniles. Glycogen, free glucose and protein concentration was significantly higher (92, 82, 32 %, respectively) ($p<0.05$) in muscle of early pre-juveniles, compared to late pre-juveniles (Fig. 4a, 4b and 4d). Triglycerides concentration was similar (Fig 4c).

4 Discussion

In the present paper we analysed, for the first time, the digestive and metabolic profile at the biochemical level of pre-juveniles of *M. liza* inside a coastal lagoon after recruitment. Our results show that early and late pre-juveniles from MCh exhibit a battery of digestive enzymes in the intestine that support their feeding habit (iliophagous). The existence of specific digestive enzymes (as being a link between digestion and absorption of nutrients) is generally related to the nature of the dietary components potentially used in metabolic processes (Cadiz et al., 2018; Calí et al., 2019; Faulk and Holt, 2009; Guo et al., 2019; Karasov and Douglas, 2013). The digestion of key glycogenic substrates (e.g. starch/glycogen) by amylase and membrane-bound disacharidases in the intestine is a main source of circulating glucose in fish (Bakker et al., 2011; Gominho-Rosa et al., 2015; Polakof et al., 2011, 2012; Steimberg, 2018). This is important for maintaining glucose homeostasis and, therefore, to support regular functions and responses to environmental

stresses (Chen et al., 2018; La Fleur et al., 2014; Polakof et al., 2011, 2012). The presence in the intestine of amylase and membrane-bound-disacharidases (maltase and sucrase), point out the ability of pre-juveniles of *M. liza* from MCh for complete digestion of dietary glycogenic items. The presence of lipase activity in intestine of pre-juveniles of *M. liza*, indicates the ability for dietary lipid digestion and their potential use for metabolic processes (Casas-Godoy et al., 2012; Karasov and Douglas, 2013; Steimberg, 2018) at early stages. The occurrence of key endo and membrane-bound ecto-proteases (e.g. trypsin/APN) in the intestine are an index of the proteolytic digestive capacity (Bakker et al., 2011; Steimberg, 2018). The existence of trypsin and APN activity (involved in final protein digestion) suggests the potential ability of pre-juveniles of *M. liza* from MCh for complete hydrolysis of dietary protein. The ontogenetic appearance of digestive enzymes activities are index of functional development of the digestive tract and digestive abilities of the individual (Kumar Pradhan et al., 2013). The existence of activity of membrane-bound enzymes indicates that early pre-juveniles of *M. liza* have reached intestinal maturity and the ability to carry out extracellular final dietary glycogenic carbohydrate and protein digestion (Holt, 2011; Tang et al., 2016; Tran et al., 2011).

The knowledge about biochemical characteristics (e.g. response to pH, temperature and kinetic parameters) of digestive enzymes of pre-juveniles in the wild is lacking. The maintenance of the activity of various digestive enzymes in the intestine over a wide range of pH, temperature and the Michaelis-Menten kinetics has been described in various fish

under controlled conditions (del Valle et al., 2016; Gioda et al., 2017; Huo et al., 2019; Kuz'mina et al., 1996). The tolerance to a wide range of temperatures is one of the characteristics of *M. liza*, that supports its potential use in aquaculture. In MCh, *M. liza* were recorded exposed to a wide range of water temperature (5-25ºC), denoting the thermal plasticity of this species (Acha, 1990; González-Castro, 2007; González-Castro et al., 2009b; Castellini et al., 2019). The fact that amylase, maltase, sucrase, lipase, trypsin and APN were active over a wide range of temperatures would indicate their thermotolerance in the intestine of pre-juveniles of *M. liza*. This characteristic could allow the maintenance of the capability for digestion of glycogenic carbohydrates, lipid and protein and, then, to sustain key physiological functions under distinct environmental temperatures. This could be important for *M. liza* inhabiting zones II and III of MCh (González-Castro et al., 2009b), which exhibit higher temperatures compared to those of coastal water. Particularly, active digestive enzymes at high temperatures could ensure an adequate supply of the resulting metabolites to support the growth during summer, when water temperature increases due to the shallow depth of the inner zones of MCh.

The higher amylase, sucrase, trypsin and APN specific activities in late pre-juveniles of *M. liza*, shown in the present work, suggested the occurrence of digestive adjustments at the biochemical level in relation to age, which could lead to an increased ability for glycogenic carbohydrates and protein digestion. Unlike sucrase, similar maltase activity was found in early and late pre-juveniles. This suggests that this activity could correspond to a sucrase-

independent maltase-glucoamylase complex (Bertucci et al., 2018; Brun et al., 2020; Chaudet et al., 2019; Dhital et al., 2013; Morelos-Castro el at., 2020). Furthermore, the potential increased ability for final glycogenic carbohydrates digestion could be mainly sustained by sucrase activity in late pre-juveniles. The similar lipase specific activity in the intestine of early and late pre-juveniles suggests that changes in dietary lipid digestion would not be an adjustment at the biochemical level in relation to age. The fact that lipase and maltase specific activities were similar in both sub-stages of pre-juveniles (while those of the other digestive enzymes determined increased) suggests that specific refined mechanisms of regulation are involved in digestive adjustments related to a short-term age variation. Amylase activity has also the ability to digest glycogen in various animals (Asaro et al., 2017). Therefore, a high amylase activity in the intestine of carnivorous and omnivorous fish enable them to take a higher advantage of nutrients via the hydrolysis of glycogen from items of animal origin (Uscanga-Martínez et al., 2011). This could be the case for pre-juveniles of *M. liza* from MCh. The values of IC found in the present work of early (2,06) and late (3,04) pre-juveniles of *M. liza* are in accordance with an iliophagous diet (Brusle, 1981; Cardona, 2016; Castellini et al., 2019; Karachle & Stergiou, 2010) and corresponded to those found for estuary-recruited pre-juveniles (Acha, 1990). The IC (and number of pyloric cacae) are commonly employed in Mugilidae as index of the digestion efficiency (Whithfield, 2016). Marais (1980) showed that *M. cephalus* has an IC approximately two times higher when compared with *Liza tricuspidens* (Smith 1935), *L. richardsoni* (Smith 1846) and *L. dumerili* (Steindachner 1870) of the Swartkops estuary,

although by the opposite, the former species presented the fewest and most poorly developed pyloric caeca. According to Odum (1970), it can be suggested that increased IC of *M. cephalus* would be necessary in this species for complete digestion of detrital material and diatom algae, in the absence of large number of well-developed pyloric caeca. Despite the changes in the diet of young mullets (zooplanktophagous to iliophagous) that occurs in the surf zone, the observed differences in IC and digestive capacity at the biochemical level between early and late pre-juveniles strongly suggest that a continuous post-recruitment adaptation to its new feeding habits occurs inside the estuary.

The levels and types of energy reserves are an expression of the metabolic characteristics of an animal (del Valle and López Mañanes, 2012). Both liver and muscle of late pre-juveniles of *M. liza* from MCh appear to be glycogen storage sites. The higher free glucose content in liver than in muscle suggests a main role for the liver, probably in the maintenance of an adequate and sustained glucose supply. Under controlled conditions, juveniles of this species store glycogen in liver (Lisboa et al., 2015; Ramos et al., 2015). In early pre-juveniles, muscle also appears to be a site of glycogen storage. Since we could not determine energy reserves in the liver, whether this organ has a role in carbohydrate storage (or of other energy substrates) remains to be investigated. In various fish, lipids are used as sources of energy for the maintenance of various physiological processes, growth, reproduction, migration and movement (Sandre et al., 2017; Steimberg, 2018; Tocher, 2003). After feeding, excess fatty acids are exported as lipoproteins from the liver and

stored as triglycerides in specific sites (Ballantyne, 2014; Grosell et al., 2011). Adult mullets possess not only liver and muscle storage of lipids, but also an important site of accumulation of lipid tissue, which is commonly known as “retroperitoneal fat”. This tissue becomes particularly evident in adult individuals of *M. liza*, captured in April-May, prior to their reproductive migration (González-Castro 2007; González-Castro et al., 2009a). The fact that no retroperitoneal fat was found in both pre-juvenile stages suggest the role of other organs in triglyceride storage. This appears to be the case for muscle in late pre-juveniles of *M. liza*. The storage of triglycerides in muscle occurred in several fishes, and can explain a large proportion of the total reserves (Peng et al., 2015; Tocher 2003; Xie, 2017). The lower triglyceride content in the liver of late pre-juveniles suggests that this organ could be a source for storage and mobilization of these reserves. Since the lipid digestion–absorption routes are unknown in *M. liza*, further experimental approaches are needed to establish the possible interaction between lipase activity in the intestine and metabolic pathways in energy storage tissues. Similar to late pre-juveniles, the muscle also appeared to be a site for triglyceride storage in early pre-juveniles. Both liver and muscle appeared to be sites for protein reserves in late pre-juveniles of *M. liza* and muscle also for early pre-juveniles from MCh. These findings suggest a link between APN activity in the intestine and adequate absorption of amino acid to be used in protein storage building**.** In mammalian intestine, APN forms complexes with neutral amino acid transporters influencing amino acid absorption (Fairweather et al., 2012).

In summary, the occurrence of amylase, maltase, sucrase, lipase, trypsin and APN activities in the intestine and the profile of energy reserves suggest that pre-juveniles of *M. liza* from MCh exhibit an adequate digestive battery to potentially perform complete hydrolysis of various dietary substrates, supporting feeding and growth inside the estuary after recruitment. Our results represent an important contribution that increases knowledge of the biochemical and ecophysiology of pre-juveniles (Querimana) of *M. liza* in particular, and of Mugilidae in general.

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Figure captions

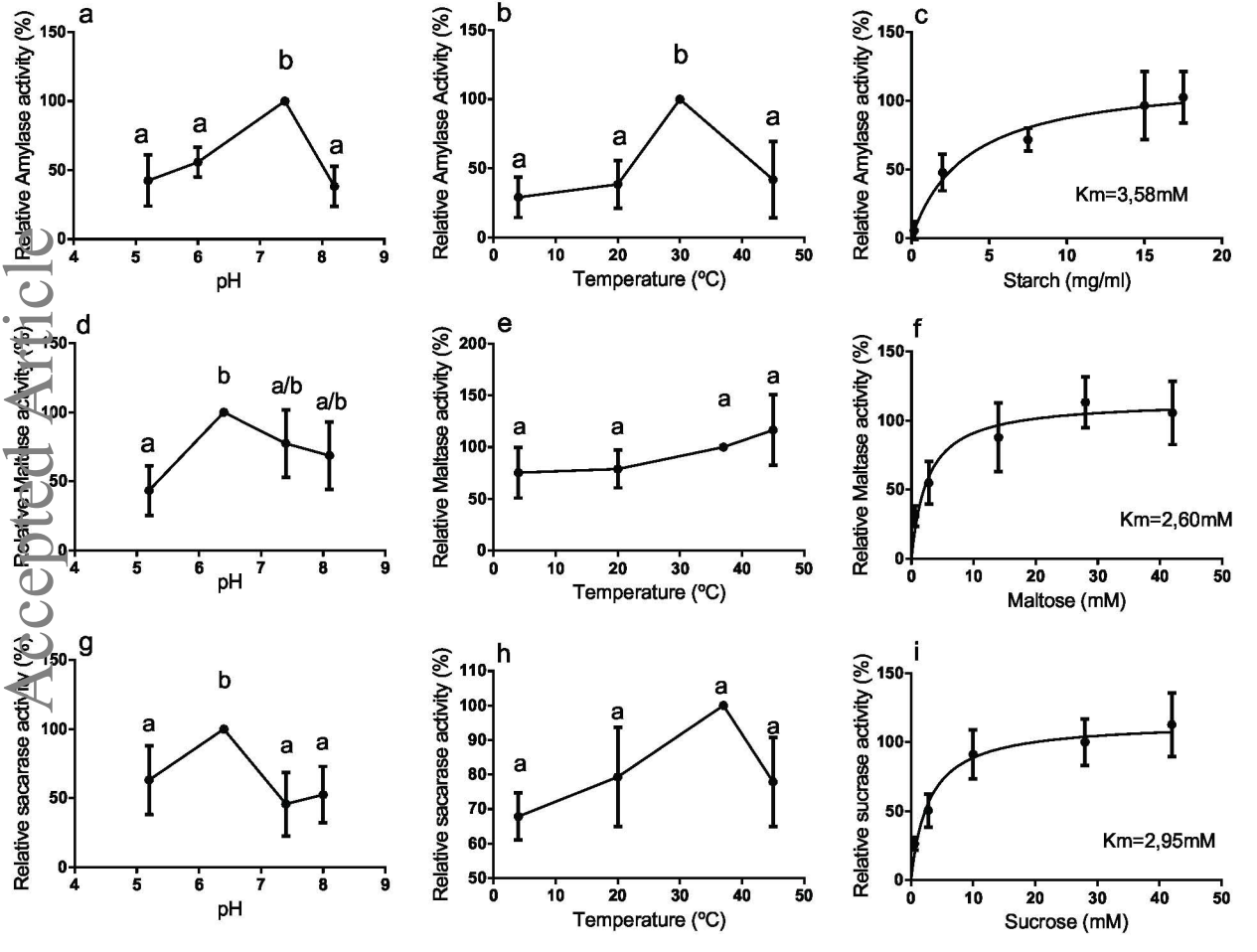
Figure 1. Partial characterization of amylase, maltase and sucrase activities in intestine of late pre-juveniles of *Mugil liza.* Effect of pH (a), temperature (b) and starch concentration (c) on amylase activity. The values are expressed in relation to the activity at pH 7.4, 30°C and 15 mg ml^{-1} starch, respectively (100%). Effect of pH (d), temperature (e) and maltose concentration (f) on maltase activity. Effect of pH (g), temperature (h) and sucrose concentration (i) on sucrase activity. The values of maltase and sucrase activity are expressed in relation to the corresponding activity at pH 6.4, 37°C and 28 mM sustrate, respectively (100%). Data are the mean ± S.E. for ten individuals. Different letter indicates significant differences.

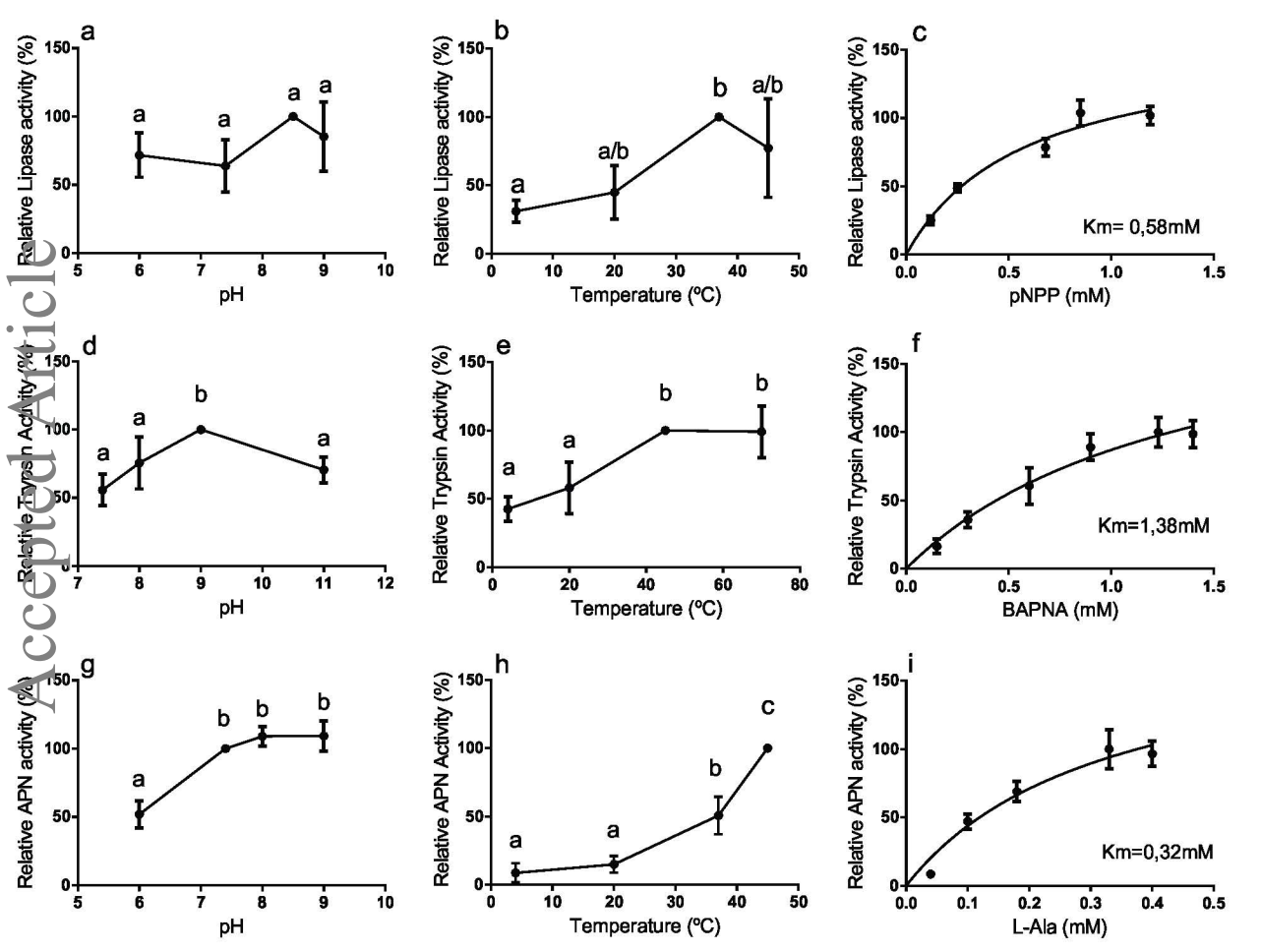
Figure 2. Partial characterization of lipase, trypsin and APN activities in intestine of late pre-juveniles of *Mugil liza*. Effect of pH (a), temperature (b) and pNPP concentration (c) on lipase activity. The values are expressed in relation to the activity at pH 8.5, 37°C and 0.87 mM pNPP, respectively (100%). Effect of pH (d), temperature (e) and BAPNA concentration (f) on trypsin activity. The values are expressed in relation to the activity at pH 9.0, 45°C and 1.23 mM sustrate, respectively (100%). Effect of pH (g), temperature (h) and L-Ala pNA concentration (i) on APN activity. The values are expressed in relation to the activity at pH 7.4, 45°C and 0.4 mM sustrate, respectively (100%). Data are the mean ± S.E. for ten individuals. Different letter indicates significant differences.

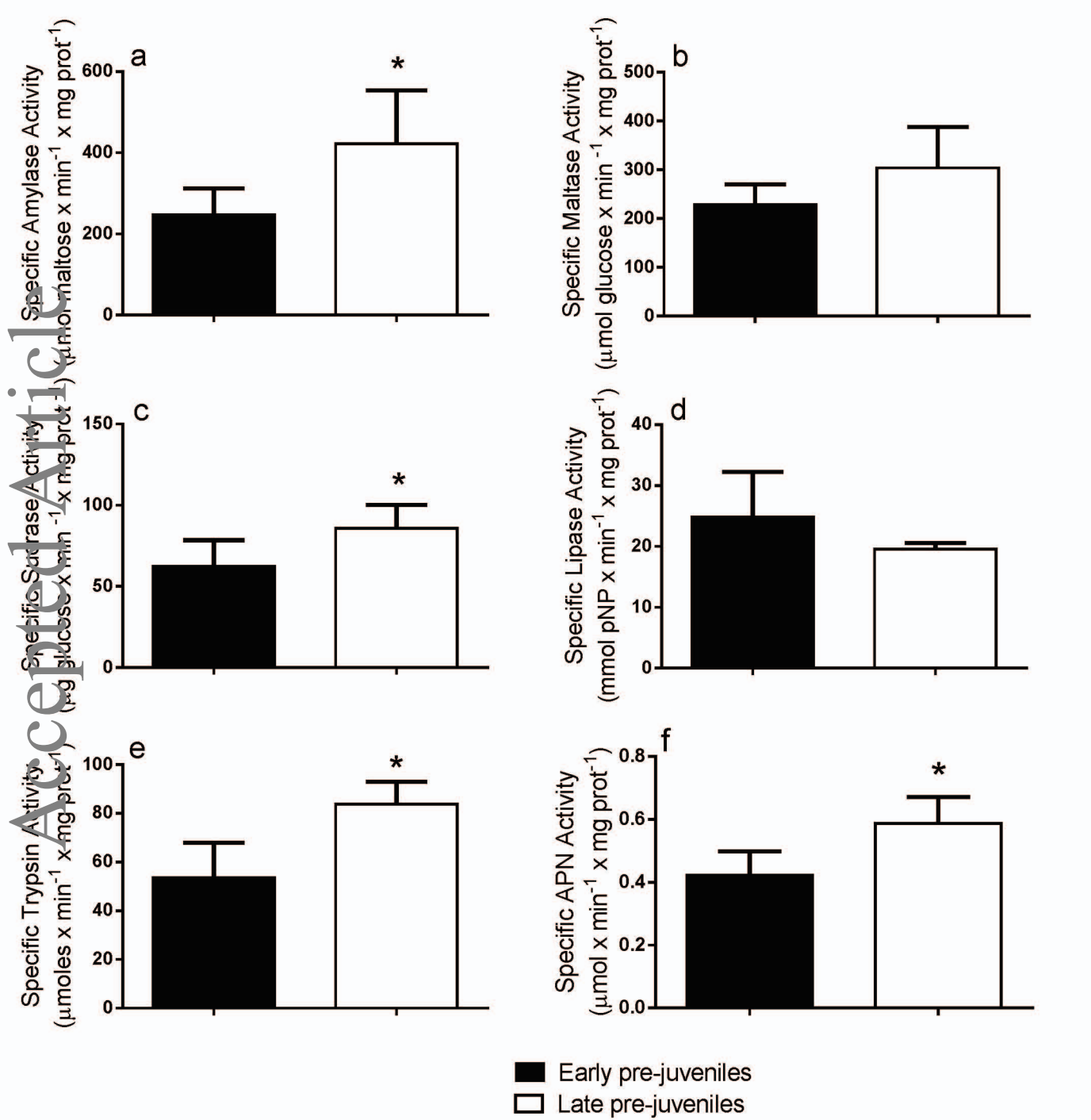
Figure 3. Amylase, maltase, sucrase, lipase, trypsin and APN specific activity in the intestine of early and late pre-juveniles of *M. liza*. The asterisks indicate difference

from the corresponding value in early pre-juveniles ($P < 0.05$). Data are the mean ± SE for ten individuals for each stage.

Figure 4. Glycogen (a) free glucose (b), triglycerides (TAG) (c), and protein (d) concentration in liver and muscle of early and late pre-juveniles of *M. liza*. The asterisks indicate difference between organ ($P < 0.05$). Data are the mean ± SE for ten individuals for each stage.







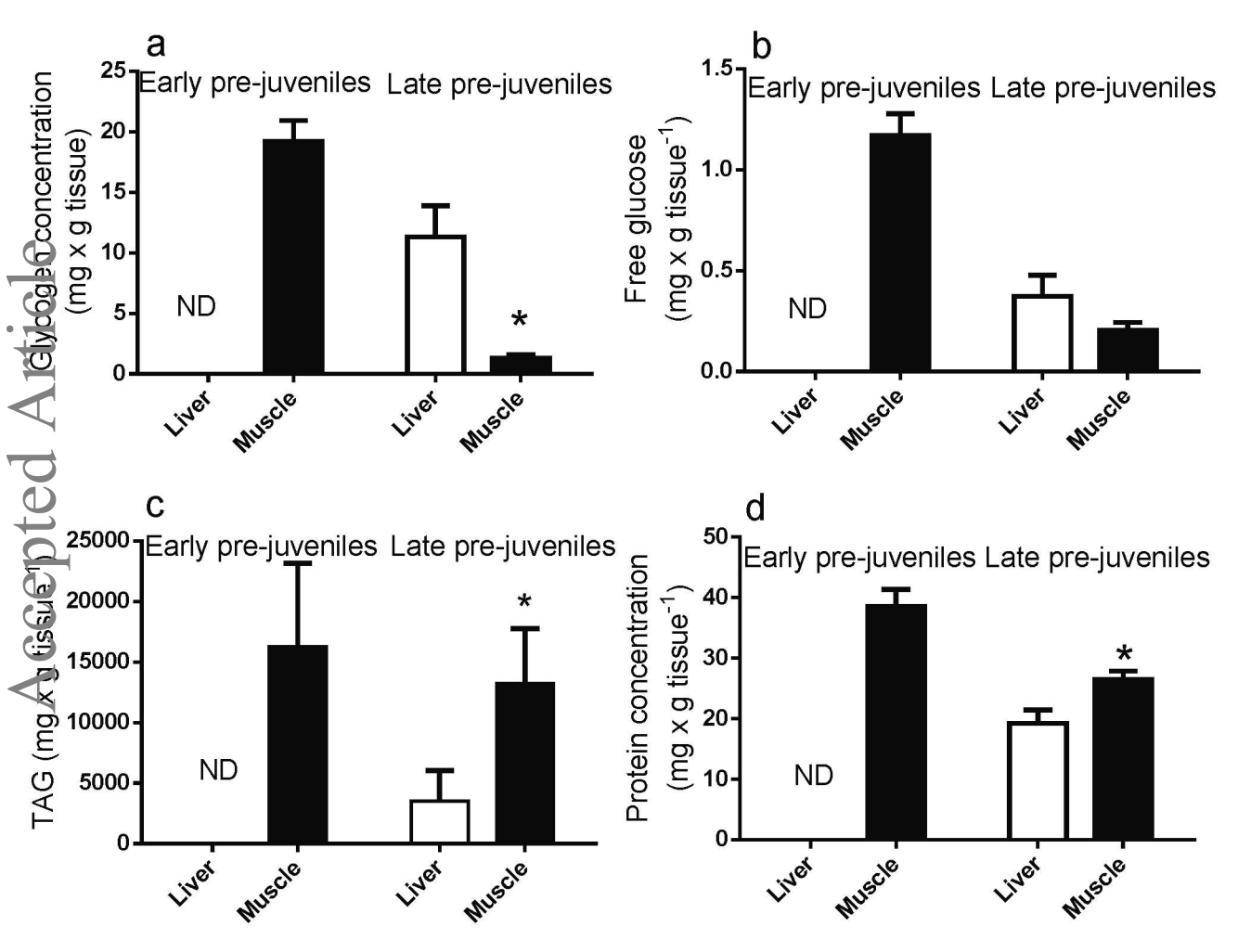


Table 1. Total length (TL), standard length (SL), total weight (TW), intestinal coefficient (IC), hepatosomatic index (HSI) and retroperitoneal fat of early and late pre-juveniles of *M. liza* recruited in Mar Chiquita Coastal Lagoon. Mean: M; range: Rg; standard deviation: (SD); number of samples: N. *The asterisks indicates significantly different from the corresponding value in early pre-juveniles ($p < 0.05$).

	TL (mm)			**SL (mm)**			**TW (g)**			**IC**			**HSI (%)**			**RF(g)**	**N**
	M	Rg	SD	M	Rg	SD	M	Rg	SD	M	Rg	SD	M	Rg	SD		
Early pre-juveniles	36,08	26-40,5	4,29	30,05	23-33	3,44	0,34	0,1-0,5	0,13	2,06	1,78-3,21	0,38	2,06	0,3-3,3	0,96	0,00	10
Late pre-juveniles	50,8*	45-56	3,19	42,8*	38-47	2,78	1,74*	1-1,9	0,29	3,04*	2,22-3,63	0,52	1,96	0,44-2,9	0,68	0,00	10