Research Article Time-dependent changes of alkaline, neutral, and acid protease activities, molecular weight distributions, and proximate compositions of live feeds

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Abstract

In this study, alkaline, neutral, and acid enzyme activities, molecular weight distributions, and proximate compositions of live feeds were determined in a time-dependent manner. Samples were taken from three different live feeds and Artemia cysts. R refers to rotifer, A-0 refers to non-enriched Artemia / A. nauplii, and A-1 refers to enriched Artemia / A. metanauplii. Sampling was done at 0th, 6th, 12th, and 18th h (T) and before enrichment as R (R-0) and A1 (A1-0). Depending on time, while enriched rotifers have high acid enzyme values at pH 3 and 4, neutral and alkaline enzyme values of enriched Artemias were higher (p < 0.05). Time-dependent decreases were detected in the enzyme activities of enriched Artemia and rotifers. Live feeds enriched for up to 12 hours should be used with a molecular weight fraction of 2,532 Da \geq (p<0.05). When marine finfish such as European sea bass and gilthead sea bream are started feeding, their digestive enzymes have alkaline and then acid characteristics. On the other hand, in this study, it was determined that rotifer enzymes had higher acid protease activities and A. metanauplii had higher alkaline protease enzyme activities. Considering this situation, A. nauplii should be given together with rotifers and A. metanuplii should be started as soon as possible. In addition, considering the enzyme activity values of the rotifers used as the first feed, the use of rotifers that are not enriched in terms of the contribution of live feeds to the larval digestive system should also be taken into account.

Keywords: Protease, Molecular weight, Proximate, Rotifer, Artemia, Live feed

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Introduction

Although rotifer and Artemia taxa are not widely found in natural food areas, they are widely used in the feeding of marine finfish and crustacean larvae due to their ease of culture (Lubzens et al., 2001; Kanazawa, 2003; Izquierdo, 2004; Palmtag et al., 2006; McKinnon et al., 2009; Oie et al., 2011; Gonçalves et al., 2014; Zaleha Kassim et al., 2014; Erdogan and Ertan, 2015). B. plicatilis can be expressed as a species complex according to genetic differences and Artemia can be expressed as a species complex according to their reproductive isolation, and there may be differences in nutritional values and enzyme activities after enrichment (Leger et al., 1986; Lu and Warner, 1991; Van Stappen 1996; Hagiwara et al., 2007; Bear et al., 2008; Vasileiadou et al., 2009). The fact that the protease activities of Artemia cysts are higher in parthenogenetic strains than in bisexual strains (Lu and Warner, 1991) affect the larval rearing of marine hatcheries. In addition to this biological assessment, environmental and global climate changes such as El Niño also affect Artemia production (GSLEP, 2017; Le et al., 2018; Lindegren et al., 2018; Fuenzalida, 2019) as well as nutritional values and enzyme activities.

In the early stages of the larvae, the digestive system is not fully functional, although low gastric and digestive enzyme activities are present prior to exogenous feding (Person-Le Ruyet, 1989; Hoehne-Reitan *et al.*, 2001a,b; Zambonino Infante and Cahu, 2001; Lazo et al., 2011). Alkaline proteases are important in the first day of feeding,

while acid proteases are important in digestion towards the end of the larval stage (Lazo et al., 2011). As the weeks progress, enzyme activities begin with stimuli. independent dietarv of ontogenesis, depending on the food ingested (Hoehne-Reitan et al., 2001a.b: Kolkovski, 2001; Lazo et al., 2011). The maturation process of the digestive system can be damaged if the larvae are fed with diets that are not suitable for their needs (Zambonino Infante et al., 2008). In this regard, live feed is an important essential food for newly hatched and immature digestive systems and enzyme-deficient finfish and shrimp larvae (Zaleha Kassim et al., 2014). Determining the molecular weight distributions of the nutrients used in aquaculture as well as the proximate nutritional compositions of rotifer and Artemia provided important approaches to the evaluation of nutritional results depending on the enrichment and enriching product (Westelmajer, 2008; Beyhan, 2011; Radhakrishnan et al., 2020). Molecular weight distributions were calculated to determine soluble digestive products, peptide distributions and leaching status of microdiet, protein sources of diets and use of feed ingredients, and status of live feeds (Grabner, 1985; Alarcon et al., 1997; Langdon, 2003; Carvalho et al., 2004; Lemos et al., 2004; Kvåle et al., 2006; Langdon et al., 2007; Onal and Langdon, 2009; Diken, 2018; Yenmiş and Naz, 2018). Besides, in many studies, protease activities of rotifers and Artemia were determined in pH 8.5 substrate (Naz, 2008; Naz et al., 2011; Naz and Yufera, 2012; Hakozu, 2014; Diken et al., 2016a, b, 2019). It has been reported that no further studies have taken place on changes in protease enzyme activity over time and hatchery practices of live feeds on different pH substrates. In this study, changes in alkaline, neutral, and acid protease enzyme activities, molecular weight distributions, and proximate nutritional compositions of rotifers and Artemia in commercial live feed cultures were determined depending on storage at +4°C from before and after enrichment to use. Evaluation of these factors affecting the larval weaning success of larvae together will contribute to the literature and practices.

Materials and methods

Culture of live feeds

The live feed culture was carried out at the EGEMAR Aquaculture Food Industry and Commercial Incorporated Company (Aydın, TÜRKİYE). The live feed culture and sampling protocol are shown in Table 1. For this purpose, samples were taken from three different live feed (R refers to rotifer, Brachionus plicatilis; A0 refers to non-enriched Artemia/A. nauplii; A1/1, and A1/2 refers to enrihed Artemia/A. metanauplii) and Artemia cysts. The live feeds' sampling was made before enrichment (before enrichment; 0, rotifer; R-0 and A. metanauplii; A1-0), and at 0th (A0's after hatching cysts harvest and A1's after enrichment harvest), and stored at $+4^{\circ}C$ at the 6^{th} , 12th, and 18th h(T=hours; T0, T6, T12, and T18) after enrichment (R, A1), and

A0's after harvest. The live feeds were shocked in ice water, then washed with +4°C tap water and rinsed with distilled water. Samples were stored in an Air Liquide GT 40 liquid nitrogen tank at -196°C. After the field study, samples brought to the laboratory were kept in the Daihan SimpleFreez U400 freezer at -80°C until analysis. Therefore, this study does not need ethical approval as it included invertebrate groups.

Rotifer, B. plicatilis (R) culture (Table 1); Rotifers were fed with Algamac Protein Plus (Aquafaune Bio-Marine Inc. Hawthorne USA) at a rate of 0.4-0.6 g L million⁻¹. The stocking rate of rotifers is 462 prey mL⁻¹ and the harvesting rate is 1,158 prey mL⁻¹. The sample was taken from the rotifers after being harvested and washed at 72nd hour before enrichment at 74.5th hour (R-0). Rotifers with an enrichment stock ratio of 972 prey mL⁻¹ were pre-fed with Ebio FB (Ege Biyoteknoloji A.Ş. İzmir-Türkiye) at 0th and 7th hours. They were enriched with Spresso (Aquafauna Bio-Marine Inc. Hawthorne USA) at a rate of 160 g/m³ at the 13^{th} and 16^{th} hours. The first sample was taken from enriched rotifers harvested and washed at the 19th hour after enrichment in 21.5th hours (R-T0) and stocked at 70 million prey L⁻¹ for larvae feeding at $+4^{\circ}$ C. Samples were collected at the 6th (R-T6), 12th (R-T12), and 18th (R-T18) hours following the enrichment.

non-enriched *Artemia/A*. nauplii (A0) culture (Table 1); After hydration and decapsulation, *Artemia* (Artemia Cysts, Vinh Chau-Bac Lieu Artemia Co. Op) cysts were stocked at a rate of 0.9 g L⁻¹.

Hatching took place in 17 hours and the total amount was 76 million. After harvesting and washing, the first sample was made at 18.5th hours without transferring to larvae (A0-T0) and the remaining samples were stored at +4°C for 5 million prey L⁻¹ for larvae feeding. A0-T6, A0-T12, and A0-T18 samples were made at the 6th, 12th, and 18th hours following the first sampling, respectively. In addition. before decapsulation (A0-E) and after decapsulation (A0-D) samples were taken from Artemia cysts used by decapsulation.

Enriched Artemia/A. metanauplii (A1) culture (Table 1); After hydration, Artemia (Artemia EG, Artemia SepArt EG>225,000 npl/g INVE Aquaculture Salt Lake City Utah/USA) cysts were stocked in 2 tanks at a rate of 2.1 g L^{-1} . Hatching took place in 24 hours and the total amount in 2 tanks was calculated as 3,115,000,000 nauplii. Following harvesting and washing, samples were taken before enrichment at 26.5th hour (A1-0). Artemia enrichment was made with two different products, Spresso (1 kg ton⁻¹) and Red Algamac-Red AMC (Aquafauna Bio-Marine Inc. 4643 W. Rosecrans Ave Hawthorne California 90250 USA) (1.4 g/per million Artemia) at 0th and 11.5th hour. Harvesting and washing were started at the 19.5th hour, and samples were taken from enriched Artemias before transfer to larvae at the 21.5th hour (A1/1 & A1/2-T0). For the larvae feeding, Artemia was stocked at the rate of 10 million prey l^{-1} at +4 °C and was sampled at the 6th (A1/1 & A1/2-T6), 12th (A1/1 & A1/2-T12), and 18th (A1/2) hours. *Artemia* cysts were also sampled (A1-E).

Alkaline, neutral, and acid protease enzyme activities of live feeds

Protease enzyme activities were determined by extracting live feeds. Time-dependent enzyme activities of live feeds were determined according to acid (pH 3, 4, 5, 6), neutral (pH 7), and alkaline (pH 8.5) protease groups.

Extracts of live feeds

The live feeds were rinsed in distilled water after thawing, and then extracts of live feeds were prepared by homogenization (Daihan, WiseTis HG-15D) with distilled and water centrifuged at 16,000 G for 30 minutes at +4°C (Sigma, 2-16 K). The resulting supernatants were protected in a -80°C freezer (Daihan SimpleFreez U400) for analysis (Diken, 2018).

Protein analysis

Soluble protein concentrations in live feed extracts were determined by a protein-dye solution developed by Bradford (1976) (Biorad Protein Assay, Cat. No: 5002). After performing Biorad kit procedures, spectrophotometer (Shimadzu, UV mini 1204)measurements were performed at 595 nm. The values obtained were used to express the protease results in U mg⁻¹ protein.

Time-day (hour)	Explanation $\pm 3E$
Dotifor Brasihonus	nlicatilis (D)
Culture	pucauus (R)
T=0(0 <24 hour) T=1(24 <48 hour) T=2(48 <72 hour)	Feeding; Algamac Protein Plus (Aquafaune Bio-Marine Inc. Hawthorne USA) V=3,200 L, 25.5 ± 0.1 °C, $\%_0 25$ mg L ⁻¹ , and 11.4 ± 0.3 O ₂
T=3(72 hour)	Harvest (ongoing washing after harvest and first sampling before transfer to enrichment. Sampling, R-0 ; from rotifer culture/non-enriched rotifer)
Enrichment	
T=0-19 hour	Pre-feeding; Ebio FB (Ege Biyoteknoloji A.Ş. İzmir TR) Enriching; Spresso $V=2,500 \text{ L}, 26.0 \pm 0.0 \text{ °C}, \%_041 \text{ mg L}^{-1}$, and $10.1 \pm 0.5 \text{ O}_{2,}$
T=19 hour	Harvest (sampling; after harvest and washing/the first sampling of before the feeding of larvae (0. hour; R-T0) and following samples of enriched rotifers stocked at +4 °C after harvest (6., 12., and 18. hour; R-T6 , R-T12 , R-T18)
Non-enriched Artema Artemia Cysts, Vinh	<i>ia</i> , <i>Artemia</i> nauplii (A0) Chau-Bac Lieu <i>Artemia</i> Co. Op Hydration (1.5 hours) and Decapsulate (chlorac - sodium hypochlorite + NaOH at 15 minute); 100 g L ⁻¹ <i>Artemia</i> ; 11% ₀ and 20.5 °C water
	V=325 L, 29.3 \pm 0.0 °C, %_041 mg L-1, and 11.5 \pm 0.2 O_2
T=17 hour	Hatching (sampling; the first sampling after harvest and washing/the first sampling of before the feeding of larvae (0. hour; A0-T0) and following samples of enriched rotifers stocked at +4 °C after harvest (6., 12., and 18. hour; A0-T6 , A0-T12 , A0-T18), cysts (A0-E) and decapsulated cysts (A0-D) of Artemia Cysts
Enriched Artemia/EO Artemia EG, Artemia Culture	<i>G Artemia</i> , <i>Artemia</i> metanauplii (A1) <i>a</i> SepArt EG >225.000 npl/g INVE Aquaculture Salt Lake City Utah/USA
T=24 hour	Hydration V=3,400 L (x2), 29.5 \pm 0.0 °C, 41% ₀ mg L ⁻¹ , 11.6 \pm 0.1 0 ₂ Hatching (ongoing washing after harvest and first sampling before transfer to enrichment. Sampling (A1-0); before enrichment <i>Artemia</i>)
Enrichment	
T=0–19.5 hour	Spresso V= 4,000 L, 26.0 \pm 0.0 °C, % ₀ 41 mg L ⁻¹ , 13.4 \pm 0.2 0 ₂ Red AMC V=325 L, 26.0 \pm 0.0 °C, % ₀ 41 mg L ⁻¹ , 11.5 \pm 0.5 0 ₂
T=19.5 hour	Harvest (sampling; after harvest and washing/the first sampling of before the feeding of larvae (0. hour; A1/1 & A1/2-T0) and following samples of enriched <i>Artemia</i> stocked at +4 °C after harvest (6., 12., and 18. hour; A1/1 & A1/2-T6, A1/1 & A1/2-T12, A1/2-T18)

Table 1: Culture of live feeds and sampling of live feeds (mean \pm SE).

Acid and neutral protease activities of live feed extracts were determined according to Anson (1938) and alkaline protease activities according to Walter (1984). Hemoglobin solution used as substrate in acid and neutral protease groups at a concentration of 5 g L^{-1} (Buffer: Citrate-Phosphate; the mixture

of 0.1 M citric acid and 0.2 M dibasic sodium phosphate solutions, pH: 3, 4, 5, 6, 7) and the casein solution used as substrate in alkaline protease groups were prepared at a concentration of 10 g L⁻¹ (Buffer: 50 mM Tris HCl, pH: 8.5). **Buffer** and larvae extracts were incubated for 30 minutes at 37°C and incubated for a further 60 minutes by adding 500 µL of hemoglobin or casein. The reaction was stopped by the addition of 0.5 mL of trichloroacetic acid (TCA at a concentration of 120 g L⁻¹). Protease activities will be given as the expression of the amount of tyrosine released per minute in μg (U mg⁻¹ protein).

Proximate compositions

Lipid content of live feeds was given according to Bligh and Dyer (1959), protein amount according to the Kjeldahl method (N×6.25) AOAC (2000a) using protein pre-combustion unit (Velp UD-20), and fully automatic protein distillation unit (Velp UDK 142), and ash content according to AOAC (2000b).

HPLC gel filtration chromatography determination of molecular weight profiles of live feeds

Molecular weight profiles were determined according to Boza et al. (1994). Samples were stirred in a phosphate buffer (pH=8.5; 10 mg mL⁻¹) and centrifuged. The supernatants obtained were passed through a 0.22 mm syringe filter and each sample extract was analyzed by HPLC-Gel Filtration Chromatography using **TSKGel** a G2000 SWXL column. The mobile phase used in the analysis is 0.1 M sodium sulfate solution prepared in 0.1 M phosphate buffer. The flow rate of the column was 1 mL minute⁻¹ and the detector used had UV light absorption of 230 nm. Molecular weight calculations were determined according to standards (from SIGMA) bovine albumin (67,000 Dalton, Da), ribonuclease A (13,700 Da), insulin chain A (2,532 Da), tyr-tyrtyr (508 Da), tryptophan (204 Da), tyrosine (181 Da), p-aminobenzoic acid (137 Da). Molecular weights were calculated as percentage in 4 different fractions based on the retention times of 67.000 the standards: Da< (protein/polypeptide), 67,000-13,700 Da (protein/polypeptide), 13,700-2,532 Da (protein/polypeptide), and 2,532 Da> (free amino acids+di/tri/oligopeptide).

Data analysis

Protease activities, molecular weight, and proximate composition of live feeds subjected to data were one-way ANOVA, and mean±standard error (SE) differences were made by Duncan test at p < 0.05 content level using SPSS (version 22) software statistical package. and proximate activities Protease composition measurements were carried out in triplicates and molecular weight measurements in duplicates.

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Results

Alkaline, neutral, and acid protease enzyme activities of live feeds

The statistical evaluation of the live feed groups was first performed in itself for each live feed. The highest protease activity of live feed rotifer was determined in the pH 8.5 alkaline group before being enriched (p < 0.05). The enzyme activities in the acid substrate of the rotifers enriched as in the timedependent with the rotifers before the enrichment were decreased from pH 3 to pH 4, from pH 4 to pH 5, and from pH 5 to pH 6, respectively. According to the substrate pH 6, an increase in the rotifers before enrichment in neutral enzyme activity and similarity or increase in the enzyme activities of rotifers other than the 18th hour after enrichment was detected. In contrast, the enzyme activities of the rotifers at alkaline substrate pH 8.5 increased relative to pH 5, pH 6, and pH 7. The decrease in substrate pH 8.5 alkaline enzyme activity of the rotifers was higher than the decrease in substrate pH 3 and pH 4 enzyme activity (p < 0.05). The enzyme rotifers activities of the before compared enrichment. enzyme to activity without transfer to larva after enrichment were reduced by 28.5%, 19.2%, and 55.5% at pH 3, 4, and 8.5, respectively. The enzyme activities of the rotifers before enrichment, compared to at the 18th hour stored at 4°C after enrichment, decreased by 30.7%, 41.6%, and 81.0% at pH 3, 4, and 8.5, respectively.

It was determined that enzyme activities of the A0 Artemia cysts

decreased after decapsulation at pH 3 and pH 4. In the first samples of the enriched Artemia/A. nauplii following harvest and washing after hatching of A0 cysts, pH 3 and pH 8.5 enzyme activities were similar but lower than pH 4 (p < 0.05). The enzyme activities of the A0 Artemias pH 4, and pH 8.5 were similar (p>0.05) and highest in the 6th hour after stock at 4 degrees centigrade. 12th and 18th hour enzyme activities were high at pH 8.5 (*p*<0.05). In short, pH 8.5 alkaline enzyme activities of the A0 Artemia at the 12th and 18th hours were higher than acid and neutral enzyme activities, particularly pH 3 and pH 4 enzyme activities. It was determined that the enzyme activities of the Artemias after hatching from decapsulated cysts increased in pH 3, pH 4 and pH 8.5 by 51.9%, 71.4%, and 97.4%, respectively. The 18th hour enzyme activities of the Artemias hatched from decapsulated cysts were increased by 34.11%, 74.0%, and 98.4% at pH 3, pH 4, and pH 8.5, respectively. No protease enzyme activity was detected at pH 8.5 in the A1 Artemia cysts. Enzyme activities of the A1 Artemia cysts were significantly higher only at pH 3 in other protease groups of the Artemias (A1-0) before enrichment after hatching. The enzyme activities of the enriched A1/1 Artemia as in the time-dependent with the A1 Artemias before the enrichment were higher at pH 4 than pH 3, at pH 6 than pH 5, at pH 7 than pH 6, and at pH 8.5 than pH 7, respectively. It was lower at pH 5 than pH 4. In contrast, the timedependent enzyme activities of the enriched A1/2 Artemias with A1

Artemias before enrichment were higher at pH 4 than pH 3, at pH 7 than pH 6, at pH 8.5 than pH 7, respectively. It was lower in the *Artemias* at pH 5 than pH 4. It was also lower in the *Artemias* at pH 6 compared to pH 5, but the enzyme activities of the pH 6 *Artemias* were higher than those of the non-enriched A1

Artemias and were similar to those of the *Artemias* at the 6th hour. The enzyme activities after the enrichment were found to be high at all sampling hours at pH 3, pH 6, pH 7, and pH 8.5 in the A1/1 *Artemias* (p<0.05) (Table 2).

T .	рН 3	pH 4	pH 5	pH 6	pH 7	pH 8.5	
Time	Rotifer, B. plicatilis (R)						
R-0	97.07±0.34 ^{b,A}	72.40±0.15 ^{d,A}	20.65±1.28 ^{lm,E}	12.63±0.59 ^{p,I}	21.31±0.4 ^{1,F}	102.76±1.48 ^{a,I}	
R-T0	69.42±1.03 ^{e,C}	58.52±0.69 ^{h,C}	$18.89 \pm 0.76^{mn,EFG}$	11.90±0.40 ^{pq,ij}	10.25±0.33 ^{q,H}	45.74±1.13 ^{i,J}	
R-T6	78.67±1.24 ^{c,B}	$58.44 \pm 0.79^{h,C}$	17.27±0.27 ^{no,FGH}	2.51±0.20 ^{s,M}	3.71±0.39 ^{s,J}	26.82±0.59 ^{k,M}	
R-T12	64.53±0.91 ^{g,D}	63.93±0.87 ^{g,B}	16.75±0.42°,GH	3.23±0.15 ^{s,M}	6.80±0.53 ^{r,I}	26.90±0.33 ^{k,M}	
R-T18	$67.31 \pm 0.67^{\mathrm{f,C}}$	$42.26{\pm}0.94^{\rm j,E}$	$15.48 \pm 0.74^{o,H}$	$6.65 {\pm} 0.0^{r,L}$	$3.76 \pm 0.54^{s,J}$	$19.53{\pm}0.54^{\rm lm,NO}$	
		Non-er	nriched Artemia (Arter	<i>nia</i> nauplii-A0)			
A0-E	12.57±0.34 ^{ghij,1}	$13.79 {\pm} 1.00^{\mathrm{fgh},1}$	1.26±0.37 ^{p,56}	2.41±0.41 ^{p,45}	1.00±0.22 ^{p,56}	0.67±0.00 ^{p,6}	
A0-D	$9.91{\pm}0.14^{jklm,2}$	7.12±0.55 ^{mno,3}	1.09±0.37 ^{p,56}	0.71±0.19 ^{p,6}	1.06±0.24 ^{p,56}	0.56±0.56 ^{p,6}	
A0-T0	20.58±1.08 ^{d,E}	24.93±2.68 ^{c,I}	7.10±0.90 ^{mno,JK}	15.42±1.00 ^{efg,H}	11.10±0.51 ^{hijk,H}	21.63±0.14 ^{d,N}	
A0-T6	7.66±0.25 ^{lmno,H}	16.58±0.61 ^{ef,J}	5.27±0.32°,K	$8.57 \pm 0.28^{klmn,K}$	$11.17 \pm 0.80^{hijk,H}$	17.07±0.56 ^{e,O}	
A0-T12	14.94±0.94 ^{efg,F}	16.15±0.86 ^{ef,J}	5.83±0.84 ^{no,JK}	11.26±0.40 ^{hijk,IJ}	15.85±1.05 ^{ef,G}	29.69±0.67 ^{b,L}	
A0-T18	$15.04{\pm}1.76^{efg,F}$	$27.33 {\pm} 3.64^{bc,KI}$	$7.83{\pm}0.56^{\mathrm{lmno},J}$	$10.49{\pm}0.18^{ijkl,J}$	$13.60 \pm 0.55^{\text{fghi},G}$	$34.82\pm0.28^{a,K}$	
		Enriche	ed Artemia, (Artemia r	netanauplii-A1)			
A1-E	13.21±0.39 ^{p,1}	9.33±0.76 ^{q,2}	2.80±0.45 ^{r,4}	2.28±0.39 ^{r,45}	1.19±0.27 ^{r,56}	*	
A1-0	2.72±0.66 ^{r,I}	43.86±0.69 ^{ij,E}	19.47±0.14°,EF	26.74±0.81 ^{m,F}	43.76±1.11 ^{ij,E}	134.99±0.32 ^{d,H}	
A1/1-T0	13.71±0.58 ^{p,FG}	38.51±0.13 ^{k,F}	21.00±0.38°,E	43.88±0.15 ^{ij,A}	68.07±1.19 ^{e,B}	366.19±1.31 ^{b,F}	
A1/1-T6	12.25±0.46 ^{p,FG}	46.22±0.12 ^{h,DE}	24.04±1.31 ^{n,D}	42.11±0.42 ^{j,B}	56.92±0.46 ^{g,D}	344.49±0.68 ^{c,G}	
A1/1- T12	$20.22 \pm 0.84^{o,E}$	$44.93{\pm}0.59^{\rm hi,DE}$	12.93±0.68 ^{p,I}	29.13±0.49 ^{1,DE}	$64.21 \pm 0.72^{\mathrm{f,C}}$	$429.69 \pm 0.97^{a,B}$	
A1-E	13.21±0.39 ^p	9.33±0.76 ^{rs}	2.80±0.45 ^{tu}	2.28±0.39 ^{tu}	1.19±0.27 ^{tu}	*	
A1-0	2.72±0.66 ^{tu}	43.86±0.69 ^j	19.47±0.14°	26.74±0.81 ⁿ	43.76±1.11 ^j	134.99±0.32 ^e	
A1/2-T0	19.56±0.39°,E	$28.95 \pm 0.55^{lmn,H}$	28.95±0.47 ^{lmn,BC}	36.31±1.18 ^{k,C}	80.49±1.25 ^{f,A}	461.59±1.33 ^{a,A}	
A1/2-T6	7.50±0.51 ^{s,H}	34.37±0.86 ^{k,G}	28.26±0.99mn,C	$28.07 \pm 0.35^{mn,EF}$	57.26±0.77 ^{h,D}	411.54±0.77 ^{c,D}	
A1/2-T12	11.69±1.63 ^{pr,G}	43.86±0.27 ^{j,E}	$35.38 \pm 1.12^{k,A}$	$29.92 \pm 0.63^{\text{lm,D}}$	65.81±1.21 ^{g,BC}	406.26±1.11 ^{d,E}	
A1/2-T18	7.13±1.30 ^{s,H}	48.25±0.65 ^{i,D}	31.02±1.02 ^{1,B}	17.83±1.21°,G	63.52±0.56 ^{g,C}	$417.74 \pm 1.47^{b,C}$	

Table 2: Protease activity of live feed groups (U mg⁻¹ protein, mean \pm SE).

Different superscripts show significant differences between means of protease activities (p<0.05). (R-rotifer, A0-*Artemia* nauplii, A1/1 and A1/2-*Artemia* metanauplii) are important. R-0; before enrichment is taken, R-T0, 6, 12, 18; 0, 6, 12, 18 hours after enrichment harvest, A0-E; *Artemia* nauplii cysts, -, A0-D; *Artemia* nauplii cysts after decapsulation, A0-T0, 6, 12, 18; 0, 6, 12, 18 hours harvest, A1-E; *Artemia* metanauplii cysts, A1-0; before enrichment is taken, A1/1; enriched *Artemia* metanauplii-1, A1/2; enriched *Artemia* metanauplii-2 and, A1/1-T0, 6, 12 hours after enrichment and A1/2-T0, 6, 12, 18 hours post-enrichment harvest.* There is no enzyme activity. All values belong to the values after harvest and washing.

In the A1/2 *Artemia*, pH 3, pH 5, pH 7, and pH 8.5 were found to be high at all sampling hours (p<0.05). Only at pH 6, the 6th hour enzyme activities after enrichment with the non-enriched *Artemia* were similar (p>0.05). The enzyme activities of the non-enriched A1 *Artemias* at pH 4, pH 7, and pH 8.5

after enrichment increased by 12.2%, 35.7%, and 63.1% in the A1/1 *Artemias* at the first sampling hour, respectively. In the A1/2 *Artemias*, it increased by 34.0%, 45.6%, and 70.8%, respectively. On the other hand, the increase rates in the last sampling hour were 2.4%, 31.8%, and 68.6% for A1/1 and 9.1%,

31.1%, and 67.7% for A1/2 *Artemias*, respectively.

Statistical evaluations of live feeds were made according to protease groups in Table 3. The highest enzyme activities of live feeds at pH 3 were determined in the rotifers before enrichment (p < 0.05). addition, enzyme activities of In enriched rotifers at pH 3 were found to be significantly higher than other live feed groups (p < 0.05). After hatching, the enzyme activities of the A0 A. nauplii decreased after 6th hours due to stocking at 4 degrees centigrade and then increased over time. It was found that enzyme activities increased in the A1/1 Artemias after enrichment and decreased in the A1/2 Artemias after enrichment. Enzyme activities of the nonenriched/A0 Artemias at the 6th hour, and the enriched A1/2 Artemias at the 6th, and 18th hour had the lowest enzyme values at pH 3. The highest enzyme activities of live feeds at pH 4 were determined in the rotifers after being enriched again (p < 0.05). After hatching, the A0 A. nauplii had a similar level of first enzyme activity with 18th hour enzyme activity and was higher than 6th and 12th hour enzyme activities. The A0 A. nauplii enzyme activities at the 6^{th} and 12th hours had the lowest enzyme values at pH 4. The enriched A1/2 Artemia's 18thhour enzyme activity was higher than enriched rotifer (p < 0.05). The activity of protease enzymes at pH 5 was highest in A1/2 the enriched Artemias (p < 0.05). Enzyme activities of the A1 Artemia and rotifers which were not enriched were similar (p>0.05). Enzyme activities of the enriched A1/1 Artemia

and rotifers were similar in the first sampling after enrichment. The enzyme activities of the A1/2 Artemias were higher than enriched rotifers (p < 0.05). Protease enzyme activities at pH 6 were found to be high in the enriched Artemias (p < 0.05). After enrichment first sampling and 6th hour samples were higher in the A1/1 Artemias (p < 0.05). The enzyme activities of Artemias enriched at the 12th hour were similar in the A1/1 and A1/2 Artemias (p < 0.05). The lowest enzyme activities were detected in the enriched rotifers at 6th hours and after (p < 0.05). Similarly, protease enzyme activities at pH 7 were detected in high amounts in the enriched *Artemias* (*p*<0.05) (Table 3).

The first samples of A1/2 Artemias after enrichment had higher enzyme activities (p < 0.05). 6th and 12th hour enzyme activities were similar in both the enriched Artemia groups (p>0.05). As at pH 6, the lowest enzyme activities were also detected in the enriched rotifers at 6th hours and after (p < 0.05). At pH 8.5, the highest enzyme activities were detected in the enriched Artemias (p < 0.05) (Table 2) -. The highest enzyme value of live feeds was determined from 461.59±1.33 U/mg protein the enriched A1/2 Artemias after the first samples. The enzyme activities of the enriched A1/1 Artemias increased at the 0th and 12th hours except for the 6th hour. On the other hand, it has been found that A1/2 tended to decrease. It was determined that it increased only in the 18th hour but remained below the 0th hour. Enzyme activity decreased in the rotifers after enrichment, whereas A0

Artemia nauplii were found to increase in the 6^{th} hour and after.

DH	Live feed	ТО	T6	T12	T18
P	R-0	97.07±0.34 ^e	97.07±0.34°	97.07±0.34°	97.07±0.34 ^d
	R	69.42±1.03 ^h	78.67±1.24 ^f	64.53±0.91 ^g	67.31±0.67 ^f
2	A0	20.58 ± 1.08^{n}	7.66±0.25 ^q	14.94 ± 0.94^{mn}	15.04±1.76° ^p
3	A1-0	2.72±0.66 ^s	2.72 ± 0.66^{s}	2.72 ± 0.66^{q}	2.72±0.66 ^u
	A1/1	13.71±0.58°p	12.25±0.46 ^p	20.22 ± 0.84^{1}	
	A1/2	19.56±0.39 ⁿ	7.50±0.51 ^q	11.69±1.63°	7.13±1.30 ^r
	R-0	72.40±0.15 ^g	72.40±0.15 ^g	72.40 ± 0.15^{f}	72.40±0.15 ^e
	R	58.52 ± 0.69^{i}	58.44 ± 0.79^{h}	63.93±0.87 ^g	42.26 ± 0.94^{i}
4	A0	24.93 ± 2.68^{m}	16.58±0.61°	16.15 ± 0.86^{m}	27.33±3.641
4	A1-0	43.86±0.69 ^j	43.86±0.69 ^j	43.86±0.69 ^h	43.86±0.69 ⁱ
	A1/1	38.51±0.13 ^k	46.22 ± 0.12^{i}	44.93±0.59 ^h	
	A1/2	28.95 ± 0.55^{1}	34.37 ± 0.86^{k}	43.86±0.27 ^h	48.25±0.65 ^h
	R-0	20.65 ± 1.28^{n}	20.65 ± 1.28^{n}	20.65 ± 1.28^{1}	20.65 ± 1.28^{mn}
	R	18.89 ± 0.76^{n}	17.27±0.27°	16.75 ± 0.42^{m}	15.48±0.74° ^p
5	A0	7.10±0.90 ^r	5.27 ± 0.32^{r}	5.83 ± 0.84^{p}	7.83±0.56 ^{rs}
5	A1-0	19.47±0.14 ⁿ	19.47 ± 0.14^{n}	19.47 ± 0.14^{1}	19.47 ± 0.14^{mn}
	A1/1	21.00±0.38 ⁿ	24.04 ± 1.31^{m}	12.93±0.68 ^{no}	
	A1/2	28.95 ± 0.47^{1}	28.26 ± 0.99^{1}	35.38±1.12 ⁱ	31.02±1.02 ^k
	R-0	12.63±0.59 ^{pq}	12.63±0.59 ^p	12.63±0.59 ^{no}	12.63±0.59pq
	R	11.90 ± 0.40^{pq}	2.51 ± 0.20^{s}	3.23 ± 0.15^{q}	6.65±0.0st
6	A0	$15.42 \pm 1.00^{\circ}$	8.57 ± 0.28^{q}	11.26±0.40°	10.49 ± 0.18^{qr}
0	A1-0	26.74 ± 0.81^{lm}	26.74 ± 0.81^{1}	26.74 ± 0.81^{k}	26.74 ± 0.81^{1}
	A1/1	43.88 ± 0.15^{j}	42.11 ± 0.42^{j}	29.13 ± 0.49^{jk}	
	A1/2	36.31±1.18 ^k	28.07 ± 0.35^{1}	29.92±0.63 ^j	17.83±1.21 ^{no}
	R-0	21.31±0.4 ⁿ	21.31±0.4 ⁿ	21.31 ± 0.4^{1}	21.31±0.4 ^m
	R	10.25 ± 0.33^{q}	3.71±0.39 ^{rs}	6.80±0.53 ^p	3.76 ± 0.54^{tu}
7	A0	11.10 ± 0.51^{pq}	11.17 ± 0.80^{p}	15.85 ± 1.05^{m}	13.60±0.55 ^{pq}
7	A1-0	43.76 ± 1.11^{j}	43.76 ± 1.11^{j}	43.76±1.11 ^h	43.76 ± 1.11^{1}
	A1/1	68.07±1.19 ^h	56.92±0.46 ^h	64.21±0.72 ^g	
	A1/2	80.49±1.25 ^f	57.26±0.77 ^h	65.81±1.21 ^g	63.52±0.56 ^g
	R-0	102.76 ± 1.48^{d}	102.76 ± 1.48^{d}	102.76 ± 1.48^{d}	102.76±1.48°
	R	45.74 ± 1.13^{j}	26.82 ± 0.59^{1}	26.90±0.33k	19.53±0.54 ^{mn}
8 5	A0	21.63±0.14 ⁿ	$17.07 \pm 0.56^{\circ}$	29.69±0.67 ^j	34.82 ± 0.28^{j}
0.5	A1-0	134.99±0.32°	134.99±0.32°	134.99±0.32°	134.99±0.32 ^b
	A1/1	366.19±1.31 ^b	344.49 ± 0.68^{b}	429.69±0.97 ^a	
	A1/2	461.59±1.33 ^a	411.54±0.77 ^a	406.26±1.11 ^b	417.74 ± 1.47^{a}

Table 3: Time dependent enzyme activities of live feeds according to protease groups (U mg⁻¹ protein, mean±SE).

Different superscripts show significant differences between means of protease activities (p<0.05) (R-rotifer, A0-*Artemia* nauplii, A1/1 and A1/2-*Artemia* metanauplii). R-0; before enrichment is taken, R-T0, 6, 12, 18; 0., 6., 12., 18. hours after enrichment harvest, A0-T0, 6, 12, 18; 0., 6., 12., 18. hours after harvest, A1-0; before enrichment is taken, A1/1; enriched *Artemia* metanauplii-1, A1/2; enriched *Artemia* metanauplii-2 and, A1/1-T0, 6, 12; 0., 6., 12. hours after enrichment and A1/2-T0, 6, 12, 18; 0., 6., 12., 18. hours after enrichment harvest. Dark lines are values of live feeds before enrichment. All values belong to the values after harvest and washing.

The protease enzyme activities of the rotifers before enrichment were found to be higher than after enrichment values at all substrate pHs (Table 3). There was

the only similarity with the first sample group (0^{th}) after statistical enrichment at pH 5 and pH 6 (p>0.05). In general, it was found that the enzyme activities of

the enriched rotifers and samples stored at 4 degrees centigrade after the first harvest decreased within time. Apart from pH 3 and pH 6, the enzyme activities of the rotifers decreased at the 18th hour compared to the enzyme values of the previous hour and were the lowest (p < 0.05). Only enzyme activities at pH 5 were similar to the previous one (p>0.05) (Table 2). The 12th hour enzyme activity at pH 7 increased compared to the previous one (p < 0.05)and the 12th hour enzyme activity had the lowest value similar to the 18th hour enzyme activity (p < 0.05). After the enrichment of the rotifers, there were not manv changes in time-dependent changes in enzyme activities at pH 3, pH 4, and pH 5. Enzyme activities of the non-enriched rotifers and the A1 Artemias were found to be high in pH 3, and pH 4 in the rotifers, similar to pH 5, and high in the A1 Artemia at pH 6, pH 7, and pH 8.5. The pH 3 and pH 4 acid enzyme activities of decapsulated and non-decapsulated A0 Artemia cysts and A1 Artemia cysts were found to be higher than other enzyme groups cyst activities (p < 0.05) (Tables 2 and 3). The pH 3 enzyme activities of the A0 and A1 Artemia cysts were similar (p>0.05). The enzyme activity of the A0 Artemia cyst at pH 4 was higher than A1 Artemia cysts (p < 0.05). Enzyme activities of the A0 Artemia cysts except at pH 6 and decapsulated A0 Artemia cysts except at pH 7 decreased from pH 3 to pH 8.5. Similarly, enzyme activities of the A1 Artemia cysts also decreased from pH 3 to pH 8.5. The decapsulation process

was found to reduce the enzyme activity of cysts at pH 3, 4, and 6.

Determination of proximate compositions of live feeds

Crude ash and crude lipid values after the enriched rotifers increased compared to the rotifers before enrichment, while crude protein values decreased (p < 0.05) (Table 4). The crude ash values of the non-enriched Artemia remained in similar proportions. In contrast, crude protein values decreased (p < 0.05) while crude lipid values did not change (p>0.05). The increase in crude ash values of the enriched A1/1 Artemia was higher than that of the A1/2 Artemia. Crude protein values of the enriched Artemias were decreased. However, crude lipid values increased. All proximate rates of decapsulated nonenriched Artemia were increased, while crude ash and crude lipid values were much higher. While the crude ash and crude lipid values of the nondecapsulated non-enriched Artemia cyst and enriched Artemia cyst were high, the crude protein value was higher in the non-enriched Artemia cyst. The highest crude ash values were determined at the 6th and 12th hours of the rotifer and the lowest crude ash values were detected in both Artemia cysts. The highest crude protein values were determined at the 0th hour of the enriched A1 Artemia and non-enriched Artemia (A1-0) while the lowest values were determined at the 6th hour of the enriched rotifer, at the 12th and 18th hours of the non-enriched Artemia (A0) and the enriched A1/2 Artemia. The highest crude lipid values were also detected at the 12th hour in A1/1 and at the 18^{th} hour in A1/2 of the

enriched Artemia, while the lowest values belonged to both Artemia cysts.

Table 4: Proximate composition of live feeds (%, mean±SE).						
Live feed	Crude ash	Crude protein	Crude lipid	CP/CL		
Rotifer, B. plicatilis (R)						
R-0	8.30 ± 0.56^{h}	60.53±0.70 ^{bc}	13.21 ± 0.38^{h}	4.59±0.17°		
R-T0	15.50 ± 0.67^{bc}	46.01 ± 1.94^{hi}	$17.72 \pm 0.50^{\text{fg}}$	2.61 ± 0.18^{hi}		
R-T6	17.08±0.53ª	44.53 ± 1.89^{ij}	21.25±0.70 ^{cd}	2.10±0.13 ^{kl}		
R-T12	17.46±0.21ª	48.84 ± 0.35^{fg}	20.53±0.30 ^{de}	2.38 ± 0.02^{ijk}		
R-T18	14.86 ± 0.46^{bc}	$47.72{\pm}0.92^{fgh}$	22.23±0.03°	$2.15{\pm}0.04^{kl}$		
Non-enriched A	A <i>rtemia (Artemia</i> n	auplii-A0)				
А0-Е	3.62 ± 0.18^{i}	59.33±0.20 ^{cd}	7.25 ± 0.07^{i}	8.19 ± 0.09^{a}		
A0-D	10.93±0.31 ^{de}	60.46±0.34 ^{bc}	19.78±0.23 ^d	3.06 ± 0.04^{fg}		
A0-T0	10.77±0.50def	57.33±1.19 ^{de}	20.36±0.79 ^{de}	2.82 ± 0.05^{gh}		
A0-T6	9.53±0.13 ^{fgh}	46.84±0.69 ^{ghi}	21.11±0.30 ^{cde}	2.22 ± 0.01^{jk}		
A0-T12	8.77 ± 0.32^{gh}	45.77 ± 0.77^{hij}	20.44±0.15 ^{de}	2.24 ± 0.02^{jk}		
A0-T18	9.74 ± 0.44^{efg}	43.19 ± 0.20^{ij}	20.41 ± 0.47^{de}	2.12 ± 0.04^{kl}		
Enriched Arten	<i>nia (Artemia</i> metan	auplii-A1)				
A1-E	4.41 ± 0.18^{i}	56.42±0.79 ^e	7.46 ± 0.14^{i}	7.57±0.15 ^b		
A1-0	9.02±0.24 ^{gh}	64.35±0.30 ^a	16.50±0.41 ^g	3.90 ± 0.08^{d}		
A1/1-T0	$8.87 \pm 0.30^{\text{gh}}$	58.71±0.78 ^{cde}	17.98±0.41 ^e	3.27 ± 0.05^{f}		
A1/1-T6	11.60 ± 0.44^{d}	49.84 ± 0.52^{f}	20.21±0.36 ^{de}	2.47 ± 0.07^{ij}		
A1/1-T12	14.60±0.61 ^{bc}	49.05 ± 0.96^{fg}	27.01±0.73ª	1.82 ± 0.08^{m}		
A1/2-T0	9.77±0.19 ^{efg}	62.12±0.11 ^{ab}	17.38±0.41 ^{fg}	3.58±0.08e		
A1/2-T6	$10.66 \pm 0.80^{\text{def}}$	45.77 ± 0.08^{hij}	24.25±0.70 ^b	1.89 ± 0.06^{lm}		
A1/2-T12	14.03±0.35°	44.61±0.71 ^{ij}	23.71±0.38 ^b	1.88 ± 0.03^{lm}		
A1/2-T18	11.61±0.41 ^d	45.07±0.43 ^{hij}	26.61±0.11 ^a	1.69 ± 0.02^{m}		

Different superscripts show significant differences between means of proximate composition (p < 0.05) (Rrotifer, A0-Artemia nauplii, A1/1 and A1/2- Artemia metanauplii). R-0; before enrichment is taken, R-TO, 6, 12, 18; 0., 6., 12., 18. hours after enrichment harvest, A0-E; Artemia nauplii cysts, A0-D; Artemia nauplii cysts after decapsulation, A0-T0, 6, 12, 18; 0., 6., 12., 18. hours harvest, A1-E; Artemia metanauplii cysts, A1-0; before enrichment is taken, A1/1; enriched Artemia metanauplii-1, A1/2; enriched Artemia metanauplii-2 and, A1/1-T0, 6, 12; 0., 6., 12. hours after enrichment and A1/2-T0, 6, 12, 18; 0., 6., 12., 18. hours after enrichment harvest. CP; crude protein, CL; crude lipid.

Distribution of molecular weight of live feeds

Molecular weight values of the highest 2,532 Da> free amino acid+di/tri/ oligopeptides were determined in the rotifers after enrichment harvest and in the A1/2 Artemia (Table 5). $67,000 \le Da$ protein/polypeptide values in A0 cysts, 67,000-13,700 Da protein/polypeptide values in decapsulated A0 cysts, and at 0th and 18th hour in the A0 Artemias and 13,700-2,532 Da protein/polypeptide values at 18th hour in the A0 and A1/2

Artemias were determined. It was determined that time-dependent 2,532 Da≥ changes of the rotifers before and after enrichment were less than other live feeds. Compared to the nonenriched Artemia (A1-0), both enriched Artemias have high molecular weights of 2,532 Da2. It was determined that the molecular weight of the enriched Artemias 2,532 Da≥-was similar in A1/1 but decreased in A1/2. However, 2,532 Da \geq values at 0th and 12th hours were higher at A1/2. According to the before enrichment situation, $67,000 \le$ Da protein/polypeptide values were similar in the rotifer, while the enriched *Artemia* decreased. The molecular weight value of 67,000-13,700 Da was reduced in the enriched *Artemias* and rotifers except in the 18th hour. The value of 13,700-2,532 Da increased in the enriched rotifers and the enriched A1/2 *Artemias* at the 18th hour. The A1 *Artemia* cysts 2,532 Da \geq value was higher than A0 *Artemia* cysts. 67,000 Da \leq was higher in A0 cysts, 67,000-13,700 Da in decapsulated A0 cysts.

Table 5: Molecular weights of live feeds (%, mean ± SE).						
Molecular weight fraction (Da)						
Live feed	67,000≤ protein/polipeptit	67,000-13,700 protein/polypeptide	13,700-2,532 protein/polypeptide	2,532≥ free a.acid+di/tri/ oligopeptide	2,532≥ / 67,000≤	
Rotifer, B.	plicatilis (R)					
R-0	13.71±0.94 ^{kl}	16.47±0.60 ^g	1.95±0.07 ^j	67.86±1.64 ^b	4.98±0.46°	
R-T 0	12.95±0.121	13.47 ± 0.17^{i}	1.65 ± 0.01^{k}	71.97±0.24 ^a	5.56 ± 0.07^{b}	
R-T6	14.84±0.36 ^{jk}	14.08 ± 0.92^{hi}	1.92 ± 0.02^{j}	69.21±0.62 ^b	4.67±0.07 ^{cd}	
R-T12	14.98±0.51 ^j	13.88±0.06 ⁱ	1.95±0.01 ^j	69.24±0.61 ^b	4.63±0.20 ^{cd}	
R-T18	14.63 ± 0.27^{jk}	17.28±0.27 ^g	2.25 ± 0.04^{i}	65.98±0.48°	4.51 ± 0.12^{d}	
Non-enric	ched Artemia (Arten	<i>nia</i> nauplii-A0)				
A0-E	37.40±0.12 ^a	21.95±0.46 ^{cd}	2.55±0.06 ^{gh}	38.60 ± 0.72^{i}	1.03 ± 0.02^{k}	
A0-D	33.19±0.18 ^b	26.57±0.13ª	2.52 ± 0.01^{h}	37.99 ± 0.17^{i}	$1.14{\pm}0.01^{k}$	
A0-T0	21.92 ± 0.24^{f}	27.66±0.01 ^a	3.04±0.01 ^b	47.46 ± 0.18^{h}	$2.17{\pm}0.03^{i}$	
A0-T6	22.03±0.14 ^f	24.72±0.23 ^b	2.85±0.02 ^{cd}	50.54±0.25g	2.29 ± 0.03^{i}	
A0-T12	22.40±0.16 ^t	25.31±0.35 ^b	2.99 ± 0.07^{bc}	49.45±0.50 ^g	2.21 ± 0.04^{1}	
A0-118	30.97±0.06°	27.16±0.17ª	3.12±0.04 ^{a0}	38.96±0.35 ¹	1.26±0.01 ^k	
Enriched	<i>Artemia (Artemia</i> n	netanauplii-A1)				
A1-E	29.30±0.96 ^d	19.31±0.50 ^e	$2.59{\pm}0.09^{fgh}$	49.23±1.16 ^{gh}	$1.68{\pm}0.10^{j}$	
A1-0	27.43±0.27 ^e	22.33±0.09°	$2.78{\pm}0.00^{de}$	47.56 ± 0.32^{h}	1.73 ± 0.03^{j}	
A1/1-T0	15.58 ± 0.41^{ij}	18.50±0.10 ^{ef}	2.80 ± 0.08^{de}	63.16±0.57 ^d	4.06±0.14 ^e	
A1/1-T6	16.69 ± 0.32^{hi}	17.47 ± 0.53^{fg}	2.72 ± 0.09^{def}	63.38±0.25 ^d	$3.80{\pm}0.06^{ef}$	
A1/1-T12	18.87±0.09 ^g	15.04 ± 0.04^{h}	2.46 ± 0.03^{h}	63.82 ± 0.25^{d}	3.38±0.03 ^g	
A1/2-T0	10.67 ± 0.10^{m}	14.17 ± 0.18^{hi}	2.08 ± 0.04^{j}	73.09±0.24ª	6.85±0.09 ^a	
A1/2-T6	17.13 ± 0.37^{h}	19.02±0.03 ^e	2.69 ± 0.01^{efg}	61.28±0.36 ^e	$3.58{\pm}0.10^{fg}$	
A1/2-T12	13.41 ± 0.13^{1}	16.57±0.20g	2.53 ± 0.02^{h}	67.53±0.29 ^{bc}	5.04±0.07°	
A1/2-T18	19.95±0.20g	20.91 ± 0.32^{d}	3.21±0.04 ^a	56.54 ± 0.38^{f}	2.83±0.01 ^h	

Different superscripts show significant differences between means of molecular weights (p<0.05) (R-rotifer, A0-*Artemia* nauplii, A1/1 and A1/2- *Artemia* metanauplii). R-0; before enrichment is taken, R-T0, 6, 12, 18; 0., 6., 12., 18. hours after enrichment harvest, A0-E; *Artemia* nauplii cysts, A0-D; *Artemia* nauplii cysts after decapsulation, A0-T0, 6, 12, 18; 0., 6., 12., 18. hours harvest, A1-E; *Artemia* metanauplii cysts, A1-0; before enrichment is taken, A1/1; enriched *Artemia* metanauplii-1, A1/2; enriched *Artemia* metanauplii-2 and, A1/1-T0, 6, 12; 0., 6., 12. hours after enrichment and A1/2-T0, 6, 12, 18; 0., 6., 12., 18. hours after enrichment harvest.

Evaluation of alkaline, neutral, and acid protease enzyme activities, molecular weights, and proximate compositions of live feeds

As a result (Tables 2, 3, and 6); (i) the non-enriched rotifer at pH 3 and 4 (ii) the enriched *Artemia* 12^{th} and 18^{th} at pH 5 of A1/2, 0^{th} and 6^{th} at pH 6 of A1/1, and 0^{th}

in pH 7 of A1/1, and A1/2, A1/1 and non-enriched (A1-0) in pH 8.5 enzyme values were significant (p<0.05). The enriched *Artemia* A1/2 and after harvest the rotifer (T0) were important at the initial feeding in terms of molecular weights of 2,532 Da≥ (p<0.05) (Tables 5, 6).

pH groups	Live feed	Protease	Molecular weight (2,532≥ / 67,000≤)	Proximate (protein / lipid)
рН 3	R-0	97.07±0.34 ^A	$67.86{\pm}1.64^{b}/13.71{\pm}0.94^{kl}$	60.53±0.70 ^{bc} /13.21±0.38 ^h
pH 4	R-0	72.40±0.15 ^A	$67.86{\pm}1.64^{b}\!/13.71{\pm}0.94^{kl}$	$60.53{\pm}0.70^{bc}/13.21{\pm}0.38^{h}$
	A1/2-T12	35.38 ± 1.12^{A}	$67.53 \pm 0.29^{bc}/13.41 \pm 0.13^{l}$	44.61±0.71 ^{ij} /23.71±0.38 ^b
phi 5	A1/2-T18	31.02 ± 1.02^{B}	$56.54{\pm}0.38^{f}\!/19.95{\pm}0.20^{g}$	45.07±0.43 ^{hij} /26.61±0.11 ^a
nII 6	A1/1-T0	43.88±0.15 ^A	63.16±0.57 ^d /15.58±0.41 ^{ij}	58.71±0.78 ^{cde} /17.98±0.41 ^e
рно	A1/1-T6	42.11 ± 0.42^{B}	$63.38{\pm}0.25^d{/}16.69{\pm}0.32^{hi}$	$49.84{\pm}0.52^{f}\!/20.21{\pm}0.36^{de}$
pH 7	A1/2-T0	80.49 ± 1.25^{A}	$73.09{\pm}0.24^{a}{/}10.67{\pm}0.10^{m}$	$62.12{\pm}0.11^{ab}/17.38{\pm}0.41^{fg}$
	A1/2-T0	461.59±1.33 ^A	73.09±0.24ª/10.67±0.10 ^m	62.12±0.11 ^{ab} /17.38±0.41 ^{fg}
	A1/1-T12	429.69±0.97 ^B	$63.82\pm0.25^{\circ}/13.41\pm0.13^{\circ}$	$49.05 \pm 0.96^{\text{rg}}/27.01 \pm 0.73^{\text{a}}$
	A1/2-118	$41/./4\pm1.4/^{\circ}$	$56.54\pm0.38^{\circ}/19.95\pm0.20^{\circ}$	$45.0/\pm0.43^{\text{m}}/26.61\pm0.11^{\circ}$
pH 8.5	A1/2-10	411.34 ± 0.77^{5}	$01.28\pm0.30^{\circ}/1/.13\pm0.3/^{\circ}$	$45.77\pm0.08^{-1}/24.25\pm0.70^{\circ}$
	A1/2-112	400.20 ± 1.11^{2}	$67.55 \pm 0.29^{30}/15.41 \pm 0.13^{3}$	$44.01\pm0.71^{3}/23.71\pm0.38^{\circ}$
	A1/1-10	$300.19\pm1.31^{\circ}$	$03.10\pm0.57^{\circ}/15.58\pm0.41^{\circ}$	$58./1\pm0./8^{cac}/1/.98\pm0.41^{c}$
	AI/1-10	544.49±0.08°	$03.38 \pm 0.25^{\circ}/10.09 \pm 0.32^{\circ\circ}$	$49.84\pm0.32^{2}/20.21\pm0.36^{40}$
	A1-0	134.99±0.32"	47.56±0.32°/27.43±0.27°	64.35±0.30 ^{°/} 16.50±0.41 ^s
	R-T 0		$71.97 \pm 0.24^{a}/12.95 \pm 0.12^{l}$	$46.01 \pm 1.94^{hi}/17.72 \pm 0.50^{fg}$
ular weights & roximate	A1/2-T0		73.09±0.24ª/10.67±0.10 ^m	62.12±0.11 ^{ab} /17.38±0.41 ^{fg}
	A0-E		38.60±0.72 ⁱ /37.40±0.12 ^a	$59.33 \pm 0.20^{cd} / 7.25 \pm 0.07^{i}$
	A0-D		37.99±0.17 ⁱ /33.19±0.18 ^b	$60.46 \pm 0.34^{bc}/19.78 \pm 0.23^{d}$
	A0-T18		38.96±0.35 ⁱ /30.97±0.06 ^c	43.19±0.20 ^{ij} /20.41±0.47 ^{de}
	A1-E		49.23±1.16 ^{gh} /29.30±0.96 ^d	56.42±0.79e/7.46±0.14i
b b	A1-0		47.56±0.32 ^h /27.43±0.27 ^e	$64.35\pm0.30^{a}/16.50\pm0.41^{g}$
Ă	A1/1-T12		63.82±0.25 ^d /13.41±0.13 ^l	49.05±0.96 ^{fg} /27.01±0.73 ^a
4	A1/2-T18		56.54±0.38 ^f /19.95±0.20 ^g	45.07±0.43 ^{hij} /26.61±0.11 ^a

Table 6: Evaluation of live feed preferences according to enzyme value (%, mean±SE).

Different superscripts show significant differences between means of protease activities, molecular weights, and proximate composition (*p*<0.05) R-rotifer, A0-*Artemia* nauplii, A1/1 and A1/2- *Artemia* metanauplii. R-0; before enrichment is taken, R-T0, 6, 12, 18; 0., 6., 12., 18. hours after enrichment harvest, A0-E; *Artemia* nauplii cysts, A0-D; *Artemia* nauplii cysts after decapsulation, A0-T0, 6, 12, 18; 0., 6., 12., 18. hours harvest, A1-E; *Artemia* metanauplii cysts, A1-0; before enrichment is taken, A1/1; enriched *Artemia* metanauplii-1, A1/2; enriched *Artemia* metanauplii-2 and, A1/1-T0, 6, 12; 0., 6., 12. hours after enrichment and A1/2-T0, 6, 12, 18; 0., 6., 12., 18. hours after enrichment harvest.

The after enrichment (T0) values of the non-enriched *Artemia* (A1-0) and enriched *Artemia* (A1/2) in terms of crude protein values were important (p<0.05) (Tables 4, 6). The *Artemia* enriched in terms of crude lipid values at

 12^{th} (A1/1) and at 18^{th} hours (A1/2) after enrichment were important (*p*<0.05).

Discussion

The activities of protease enzymes in live feeds were generally determined in pH 8.5 substrate (Naz et al., 2011; Naz and Yufera, 2012; Haközü, 2014; Diken et al., 2016b, 2019). In this study, alkaline, neutral, and acid total protease enzyme activities of live feeds were revealed. Warner and Shridhar (1985) reported that most Artemia cysts have cysteine proteases that are inactive at alkaline pH. Similarly, before and after decapsulation, the enzyme activities of A. nauplii and non-decapsulated A. metanaplii cysts in the substrate with pH 3 and 4 were higher. Although decapsulation decreased the protease enzymes of Artemia cysts, a significant increase was observed after hatching (A0-T0). This result was similar to the study reports reporting the effect of decapsulation on the digestibility of Artemia (Garcia-Ortega et al., 1998; Hekimoglu et al., 2014).

Yufera et al. (2000) determined that the acid protease activity of rotifers was high and the contribution of rotifer proteases to the larval gut was not significant. In the results of the study, it is seen that enriched rotifers have high acid values. However. protease unenriched rotifers (R-0) have higher alkaline protease activities. This means that rotifers to be used without enrichment will contribute more exogenous enzymes to the alkaline larval digestive system. The present study results differ from the Munilla-Moran et al. (1990) report, which reported that the enzyme activity of rotifers was lower than that of Artemia

and copepods. Compared to Artemia groups, rotifers had higher acid protease enzyme activities in pH 3 and 4 substrates. Both the increased protease enzyme activity in alkaline protease activities of rotifers and the increase in alkaline enzyme values in Artemia groups were similar to the highest enzvme increase at alkaline рH expressed by Warner (1987). The differences in the enzyme activities of Artemia metanauplii enriched according to different enrichments in the study were similar to the result reported by Munilla-Moran et al. (1990) that the enzymatic activity varies depending on the nutritional status and developmental stage. According to acid protease (pH 3 and 4) activities in rotifers, and alkaline protease activities in Artemia groups, the increase in enzyme activities as a result of enrichment supports the result reported by Naz (2008) that the exogenous enzyme contribution of live feeds will increase after enrichment. Warner et al. (1995), reported that protease activities increased after hatching in A. franciscana depending on the incubation medium. However, the present study showed fluctuations in protease enzyme activities due to storage at 4 degrees centigrade. Enzyme values increased in one group of enriched Artemias and decreased in the other group. The increase in protease activity of A. nauplii at the end of 25 hours (Garcia-Ortega et al., 1998), was similar to the trend of increase in acid (pH 3), neutral, and alkaline enzyme activities at the end of the 18th hour of the study. In contrast in this study, the excess amount of acidic cysteine protease of *Artemia* (Warner and Shridhar, 1985; Warner *et al.*, 1995) was much lower than neutral and acid protease enzyme activities due to the low activity of acid proteases. According to Naz *et al.* (2021), different fluctuations were detected with the results of the study in the changes in alkaline, neutral and acid protease enzyme activities of *Artemia* enriched with different products. The results of the enrichment used in live feed enrichment on the enzyme activities of live feeds.

The results of the studies of other researchers including the protease enzyme values in the pH 8.5 substrate were discussed with the total protease activity (T0) pH 8.5 substrate values of the current study. Naz (2008), found that the amount of trypsin increased during fasting, especially in A. metanauplii, and found that trypsin decreased during fasting in the enriched rotifer, although it was found higher than in enriched rotifers. In the present study, the total amount of protease was similarly reduced and significantly lower than enriched Artemia. However, contrary to Naz (2008), the fluctuations of the total protease content of the Artemia stored at 4 degrees centigrade in the first enrichment and the decreases in the second enrichment reveal the difference in the live feed culture of the present study. Naz et al. (2011), the protease activity value non-enriched of $(114.26\pm20.19 \text{ U mg}^{-1} \text{ protein})$, the enriched rotifers (139.42±18.38 U mg⁻¹ protein), A. metanauplii (481.31±22.10

U mg⁻¹ protein) and A. nauplii (253.48±6.54 U mg⁻¹ protein) were found to be higher than the results of the present study. According to Naz and Yufera (2012), the protease activity value of A. metanaupli (414.5±0.41 U mg⁻¹ protein) was found to be lower than the second enriched value of the present study and higher than the first enriched Artemia value. The rotifer (156.25±0.09 U mg⁻¹ protein) protease activity value was higher than the protease value of the present study. Hakozu (2014), reported that protease activity values of the enriched rotifer, A. nauplii, and A. metanauplii (U mg⁻¹ protein) were 17.98±2.82. 34.67±0.88. and 317.16±2.67, respectively. According to the present study, the rotifer and the enriched Artemia values of these results were low and the A. nauplii values were high. According to Diken et al. (2016b), the enriched Α. metanauplii's 338.02±4.65 U mg⁻¹ protein protease activity value was lower than the enriched Artemia of this study but higher than the enriched A. metanauplii value of the Hakozu (2014) report. According to the results of the protease activity values of live feeds in the present study, Diken et al. (2019), reported that the rotifer value (21.76±0.31 U mg⁻¹ protein) was low, A. nauplii value (29.3 3±0.93 and 36.00±1.48 U mg⁻¹ protein) was high, and A. metanauplii value (403.53±11.85 and 416.44 \pm 19.70 U mg⁻¹ protein) was higher than the first enriched Artemia value and lower than the second enriched Artemia value. Differences in protease activities of A. nauplii and metanauplii and an increase in protease activity of live feeds due to Artemia enrichment were reported by Pan et al. (1991), depending on the age of the Artemia hatching 12 hours after hatching increased Artemia expression of digestive enzymes supported the results of the study. The expectation of the low contribution of the Artemia to the end of the metamorphosis of the larvae of the inactive alkaline pH cysteine protease enzyme (Warner and Shridhar, 1985; Solovyev et al., 2016) was supported by the study results.

Exogenous enzyme additives will be insufficient in the feeding of early larvae that have an ontogenetically alkaline digestive system, in the use of rotifers by stocking them at 4 degrees centigrade due to their neutral and alkaline enzyme activities. According to the changes in protease enzyme activity, the use of rotifers used as the first feed without enrichment should be discussed. Considering this situation of the larvae whose digestive system has an acid character ontogenetically, the alkali enzyme contributions of Artemia are higher depending on the enrichment process. Due to the high alkaline protease activity of A. nauplii, it is important to feed A. nauplii and rotifer live feed, depending on the alkaline digestive system of the larvae. Care should be taken as storage at 4 degrees centigrade causes a decrease in the enzyme values of the rotifers. On the contrary, there is an increase in the enzyme values of A. nauplii due to storage at 4 degrees centigrade. Due to the high alkaline protease enzyme activity in enriched Artemia, it will be

important to switch to feeding the larvae with a microdiet as soon as possible. It can be used by stocking A. nauplii and metanauplii at 4 degrees centigrade until the 18^{th} hour. On the other hand, it is recommended to pay attention to the use of rotifers by stocking. While the alkaline, neutral and acid character of the digestive system is taken into account in the ontogenetic development of the larvae, this situation of live feeds should also be taken into account.

The biochemical composition of live feeds and their exogenous enzymes affect weaning success. It has been reported that biochemical the compositions of live feeds change depending the enrichment on (Westelmajer, 2008; Beyhan, 2011; Radhakrishnan et al. 2020; Pan et al., 2022). In the study, crude protein values of live feeds decreased, and crude lipid values increased depending on time. The present study results are similar to the report results, which reported that the enriched rotifer dry weight consisted of 28-63% protein and 9-28% lipid (Lubzens et al., 1989; Lubzens et al., 2001; Lubzens and Zmora, 2003; Conceição et al., 2010). Time-dependent crude protein reduction, crude lipid increase fluctuations, and crude ash fluctuations of rotifers were similar to Naz's (2008) report. However, in the report of Naz (2008), crude lipid values were high and crude ash values were low. Naz (2008), Yenmis and Naz (2018), and Aktas et al. (2019) A. nauplii and metanauplii crude ash values contain similar rates to the results of the study. Beyhan (2011) reported that Artemia had a high crude lipid value enrichment harvesting. after The fluctuations of time-dependent changes in crude lipid values of enriched Artemia were similar to Akkus (2015). Timedependent crude lipid and crude ash values of unenriched and enriched Artemia (A0 and A1) decreased in Naz's (2008) report, unlike the present study. The crude protein value of Naz (2008) was higher than the results of the study. The time-dependent crude protein value of enriched Artemia (A1) decreased depending value before on the enrichment, whereas it increased in Naz's (2008). Aktas et al. (2019) reported that the crude protein value of unenriched A. nauplii was almost twice of enriched Α. metanauplii. that However, this situation was quite different from the results of the study. Unenriched Artemia had a higher crude protein value than the current study, while the crude lipid value was much higher than the current study and the crude ash value was considerably lower. According to this study, while crude ash and crude lipid values of enriched A. metanauplii were similar, crude protein value was high.

Feeds have been reported to contain large amounts of free amino acids and oligopeptides (Lavens and Sorgeloos, 1996). In this sense, the fact that *Brachionus* have small peptides with molecular weights <1,500 Da (Ronnestad *et al.*, 2003) can be seen as an important advantage. Based on these considerations, it is an important nutritional value of live feeds to have high molecular weight distributions of 2,532 Da>, which do not show much variation due to enrichment of the study results and storage at 4 degrees centigrade. However, it is a parameter that should be observed and paid attention to due to the low value of 2,532 $Da \ge (Diken, 2018)$ in the other study. Similarly, the height of 67,000 Da \leq in Diken (2018) according to the results of the study is another important molecular weight class value to be monitored. The present study and Aktas et al. (2019), showed that enriched A. metanauplii's value of 2,532 Da≥ was similar to the second enrichment (A1/2-T0) of the present study and higher than the first enrichment (A1/1-T0). Aktas et al. (2019), had a 67,000 Da≤ value of enriched A. metanauplii, which is similar to the first enrichment (A1/1-T0) of the present study and higher than the second enrichment (A1/2-T0). According to the present study, the non-enriched A. nauplii value of 2,532 Da≥ was quite high, and the value of 67,000 Da≤ was low. A high proportion of protein in the peptide groups/ molecular weight distribution is necessary to have a balance (Carvalho et al., 2003). This equilibrium ratio based on live feed molecular weights was given in microdiets of in vitro meagre larvae (Diken, 2018). In the current study results, the equilibrium ratio of 2,532 $Da \ge / 67,000 Da \le is important in vitro$ reference in the formulation of microdiets for these evaluations. At the same time, depending on the necessity of evaluating free fatty acids in larval feeding according to (Fyhn et al., 1993),

molecular weight distributions of 2,532 Da \geq are also an important approach.

The alkaline, neutral, and acid activities. proximate enzyme composition, and molecular weight may vary with enrichment depending on the species differences and/or geographical differences of live feeds. Live feeds' storage at 4 degrees centigrade and the amount of lipid that changes with time is the result of oxidation. However, lecithin can be considered an important factor with natural and synthetic antioxidation which may prevent oxidation used in enrichment products. However, the most important factors affecting the autoxidation of oils are the type and amount of fatty acids and storage conditions such as temperature sensitivity and humidity. In of particularly lipoxygenases, the temperature and pH range are important factors. In particular, the significant decrease in pH 7 and 8.5 with the change in lipid amount after enrichment in the rotifers was related to this result. In contrast, the similarity in enzyme activities at pH 3 and 4 is indicative of this result. And for the rotifers to work at high enzymatic activity, the pH 3 and 4 acid protease groups can also be expressed as the required pH range. In other words, when the rotifers are enriched with such enriching products, they do not exhibit high enzymatic activity at the pH 8.5 required for the larva. This is indicative of the fact that the non-enriched rotifer (R-0) exhibits higher activity than enrichment at pH 8.5. This is the case in Artemia. This shows the species differences between

rotifer and *Artemia* of these enriching products that increase the lipid content of live feeds. As a result of enrichment with these enriching products, *Artemia* exhibits very high enzymatic activity at pH 8.5. Despite the increase in the crude lipid values of live feeds as a result of enrichment, the activity of the enzyme group in which the live feeds should study is insufficient depending on the age of the larvae.

In summary, the present study sheds light on the necessity of evaluating the substrate according to the pH group range in determining the enzyme activities of live feeds, and the importance of molecular weight determination of live feeds. These results, especially on the activities of enzymes ranging from alkalinity to acidity in the ontogenetic development of marine finfish larvae, will provide a more accurate assessment of the exogenous enzyme effects of live feed. Preferred molecular weight analyses as an alternative to SDS-PAGE offer a practical approach to determine the peptide structures and amino acid distributions of live feeds.

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